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Nitric oxide regulates neurite outgrowth in identified *Helisoma* neurons

By

Geoffrey Thomas Boddy

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ABSTRACT

Nitric Oxide (NO) is a diffusible gas that acts as a neurotransmitter and developmental regulator in both vertebrate and invertebrate nervous systems. We investigated the effects of NO on neurite outgrowth in identified neurons B5 and B19 from the pond snail, *Helisoma trivolvis*. Neuron B19 exhibited one population of neurites advancing at a rate of 15.2 ± 1.2 μm/hr. Application of the NO donor SNAP (100μM) to B19 caused a weak rise in cytosolic calcium concentration ($[Ca^{2+}]_c$) and an acceleration of neurite outgrowth. In contrast to neuron B19, neuron B5 exhibited two populations of neurites; slow-growing neurites advanced at 15.3 ± 0.7 μm/hr, whereas fast-growing neurites advanced at 36.9 ± 1.2 μm/hr. Application of SNAP to B5 caused a strong rise in $[Ca^{2+}]_c$, a slowing of fast-growing neurites, and an acceleration of slow-growing neurites. . Only the inhibitory effects of NO on neurite outgrowth were dependent on extracellular calcium and cyclic GMP. These results suggest that NO is an important signaling molecule in the regulation of neurite outgrowth.

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List of Abbreviations

5-HT	Serotonin
ADF	Actin Depolymerizing Factor
AA	Arachodonic Acid
CAM	Cell Adhesion Molecule
CREB	Cyclic AMP Responsive Element Binding Protein
cGMP	3', 5'-cyclic Guanosine Monophosphate
ER	Endoplasmic Reticulum
ERK	Extracellular-Regulated Protein
F-actin	Filamentous-actin
FGF	Fibroblast Growth Factor
GAP-43	Growth-Associated Protein-43
GTPases	Guanosine 5' Triphosphatases
IP3	Inositol 1,4,5 Triphosphate
MAPK	Mitogen-Activated Protein Kinase
NGF	Nerve Growth Factor
NO	Nitric Oxide
NOS	Nitric Oxide Synthase
ODQ	1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one
PLC	Phospholipase C
PKC	Protein Kinase C
SNAP	S-Nitroso-N-acetyl-D, L-penacillamine
Trk	Tyrosine Kinase Receptor

There are billions of neurons in our nervous system, and between these neurons many more billions of connections are made. The process of neuronal development is an amazing feat that requires incomparable neuronal pathfinding skills. The structure that is responsible for this is the growth cone, which is present at the tip of extending neurites and ensures that they arrive at their proper destination. The growth cone is a specialized structure that is rich in membrane receptors, making it extremely sensitive to external stimuli. Various molecular cues are distributed in extracellular patches or gradients that activate these membrane receptors and induce intracellular signaling pathways involved in growth cone advance. These signal transduction pathways act on the growth cone cytoskeleton to cause structural reorganization and subsequent changes in growth cone motility, including turning, stopping, accelerating, slowing, and branching.

The goal of our study was to determine if nitric oxide (NO), a novel neurotransmitter, is involved in growth cone function. Although neurotransmitters are best known for their actions at mature synapses, they are also required for proper neuronal development and are regulators of neurite outgrowth. Whereas typical neurotransmitters are packaged into vesicles and released by exocytosis, NO is a gaseous neurotransmitter that diffuses passively across membranes. NO has a number of effects on neuronal development and neurite outgrowth. By using a relatively simple model system, the fresh water pond snail *Helisoma trivolvis*, we sought to determine the role of NO in regulating neurite outgrowth.

Structure and function of the growth cone cytoskeleton

The growth cone contains a cytoskeleton made up primarily of actin filaments and microtubules (Forscher and Smith, 1988). The tip of the growth cone has protruding finger-like structures called filopodia, which contain bundled filamentous (F)-actin that extends proximally into the growth cone to form ribs. Between these ribs are random networks of unbundled F-actin that comprise the lamelopodium (Lewis and Bridgman, 1992). Together these structures define the distal region of the growth cone, known as the peripheral domain (Forscher and Smith, 1988). Microtubules form the structure of the proximal region, known as the central domain. These microtubules are organized into longitudinal bundles that provide structural support for the neurite. In between the peripheral and central domains lies a transition zone where microtubules and filopodial actin interact. These interactions play a central role in advancing a growth cone across its substrate.

The peripheral domain of the growth cone is a dynamic region where F-actin assembly and turnover are prevalent. Actin monomers are polymerized into filopodial bundles at the leading edge of the growth cone (Mallavarapu and Mitchison, 1999) and depolymerized at the proximal end (Forscher and Smith, 1988). These events cause F-actin to constantly turn over and advance the filopodia.

Filopodial dynamics are affected by additional complex interactions that result in a process known as retrograde flow (Lin and Forscher, 1995). As F-actin is turning over, a myosin motor anchored to the cell membrane drives the filopodial actin bundles in a retrograde direction (Lin et al., 1996). This results in an escalator-like effect, where net filopodial extension is dependent on the rate of actin polymerization and retrograde flow. When actin polymerization occurs faster than retrograde flow, a filopodium advances. In contrast, a filopodium retracts when actin polymerization is slower than retrograde flow (Lin et al., 1996). Similar events may also occur in the lamelipodium so that it too can extend and retract dynamically during growth cone pathfinding.

The rate of retrograde flow, and hence neurite outgrowth, is determined by substrate-cytoskeletal binding. When F-actin-substrate coupling increases, a "molecular clutch" is engaged that favors growth cone advance (Suter et al., 1998). Cell adhesion molecules (CAMs) found in the plasma membrane mediate this coupling by anchoring the actin cytoskeleton to the underlying substrate (Rutishauser, 1993). When an advancing growth cone encounters an attractive signal, CAMs are upregulated and bind F-actin more tightly to the substrate (Suter and Forscher, 1998). This reduces the retrograde sliding of F-actin filaments and produces the anchor needed to pull microtubules forward. The degree of substrate-cytoskeletal coupling is therefore a major determinant of the

rate of neurite outgrowth, such that growth cones tend to move across the substrate along the path of maximum adhesion.

The rates of retrograde flow and microtubule advance are influenced by a number of additional factors that affect neurite outgrowth. For example, actin-depolymerizing factor (ADF) removes F-actin from the proximal end of filopodial ribs and thus stimulates actin turnover (Bamburg et al., 1999). This process is necessary for microtubule advance, because F-actin bundles impose a steric blockade (Challacombe et al., 1996). Increasing F-actin turnover with ADF over-expression or experimentally disrupting F-actin can increase the rate of neurite outgrowth in a microtubule-based manner (Meberg and Bamburg, 2000; Marsh and Letourneau, 1984; Bentley and Toroian-Raymond, 1986). Furthermore, microtubules have been found to co-localize with F-actin in the transition zone to form a dynamic pool of microtubules (Dent and Kalil, 2001). These microtubules are thought to grow along the filopodial ribs (Kabir et al., 2001), whereby the rate of microtubule assembly must be greater than retrograde flow for a positive net movement to occur. Thus, microtubule dynamics in the transition zone are controlled by the opposing forces of F-actin-mediated steric blockade and F-actin-associated microtubule assembly.

Protein kinase C (PKC) is another factor that promotes neurite outgrowth by enhancing microtubule assembly (Kabir et al., 2001). Increased microtubule assembly coupled with pre-existing rates of anterograde transport may lead to

increased outgrowth. Alternatively, increased microtubule assembly may just increase the number of microtubules found in the transition zone, independent of a direct effect on neurite outgrowth. In this case, the additional microtubules would increase the transport of vital materials to the front of the growth cone, resulting in faster growth cone advance.

Growth cones are constantly extending the boundaries of the neurite, and as such require a steady stream of new materials. Growth cones are autonomous structures and can continue to grow for some time without any anterograde transport, even when a response requires local protein synthesis (Campbell and Holt, 2001; Zheng et al., 2001). However, anterograde transport is required to sustain neurite outgrowth over long periods of time. Myosin motors are thought to drive organelles and vesicles containing mRNA, neurotrophin receptors, and other materials along the microtubule cytoskeleton into the tip of the extending growth cone (Wang et al., 1996: Wu et al., 1997; Zheng et al., 2001; Morfini et al., 1997). Furthermore, retrograde transport of materials back to the nucleus occurs when a growth cone has encountered a substrate-associated barrier (Hollenbeck and Bray, 1987). Certain growth cone responses are dependent on transport of materials to and from the nucleus. Typically these types of responses are due to changes in the expression of growth cone regulatory proteins like ENA and VASP (Korey and Van Vactor, 2000), and are relatively slow in comparison to autonomous growth cone responses.

Growth cones are highly dynamic structures that rely on their cytoskeleton for structural stability, substrate attachment, outgrowth, and transport of materials. It is for these reasons that virtually all signal transduction pathways converge onto the cytoskeleton (Korey and Van Vactor, 2000). However, the cytoskeleton is not just composed of actin and microtubules. There are many additional proteins that interact with these main elements to orchestrate cytoskeletal reorganization. It is the activity and regulation of these many control proteins that determines growth cone behavior during neurite pathfinding.

Signal transduction elements in growth cones

There are numerous intracellular signaling pathways that act to reorganize the cytoskeleton and influence neurite outgrowth. These signal transduction pathways are regulated by two main classes of extracellular signaling molecules: soluble and substrate bound cues. Both of these influence neurite outgrowth by binding to membrane receptors, but their modes of action are somewhat different. Soluble cues occur in diffusion gradients that are sensed by the unequal activation of membrane receptors on different regions of the growth cone (Zheng et al, 2000). This in turn causes a disparate reorganization of the cytoskeleton, causing the growth cone to turn away from or towards the gradient. Substrate-bound growth cues, on the other hand, lay out a pathway for growth cones to follow. As mentioned earlier, growth cones will generally follow the

most adhesive substrate (Bray, 1970), with CAMs and F-actin-substrate coupling playing major roles in substrate dependent outgrowth.

Cell Adhesion Molecules

Cell adhesion molecules (CAMs) are a large family of proteins that include neuronal (n)-CAM, neuronal (n)-cadherin, and the L1 glycoprotein (for review see Walsh and Doherty, 1997). CAMs are membrane bound glycoproteins that help anchor neurons to the substrate and to other cells. CAMs exhibit homophilic and heterophilic binding to other CAMs, yet given these features, each one has its own unique binding properties (Meiri et al., 1998; Kuhn et al., 1991). The spatial and temporal expression of specific CAMs plays a critical role in regulating growth cone pathfinding (Schuster et al., 1996; Davis et al., 1997). For example, proper pathfinding of avian motor axons within developing muscle requires n-CAM expression during specific stages of development (Tang et al., 1992). Furthermore, motorneuron synapse formation in *Drosophila* requires increased expression of CAMs in the muscle fibers at the time of axon innervation (Schuster et al., 1996; Davis et al., 1997). Altering CAM expression during this time causes motorneurons to erroneously innervate muscles. The correct spatial and temporal expression of CAMs is also necessary for chick commissural neurons to cross the midline and accurately project to their targets (for review see Stoeckli and Landmesser, 1995). Similarly in humans, CAMs play critical

roles in neuronal development, and mutations in L1 lead to mental retardation and hydrocephalus (Yamasaki et al., 1997).

When a CAM becomes bound to an adjacent cell or substrate, it not only anchors the neuron, but can also activate intracellular signaling pathways. When bound, CAMs cluster together and activate the fibroblast growth factor (FGF) receptor, which leads to the production of diacylglycerol and a subsequent synthesis of arachodonic acid (AA)(Kolkova et al., 2000). One group suggests that AA mediates a calcium influx and thereby activates PKC (Kolkova et al., 2000), whereas, another group believes that there is no concrete evidence linking calcium influx to PKC induction (Skaper, 2001). However, both groups agree that a submembrane calcium influx (Archer at al., 1999) and PKC activity are required for CAM-mediated neurite outgrowth. PKC stimulates outgrowth by phosphorylating growth-associated protein (GAP)-43 (He et al., 1997) and mitogen-activated protein kinases (MAPKs) (Kolkova et al., 2000; Troller et al., 2001), which are both required for CAM-mediated neurite outgrowth (Meiri et al., 1998; Kolkova et al., 2000).

GAP-43 and MAPK activity are known to enhance neurite outgrowth in a number of different systems. The phosphorylation of GAP-43 in mouse cerebellar neurons stimulates neurite outgrowth (Meiri et al., 1998), whereas knocking out GAP-43 expression disrupts axon pathfinding and neuronal development (Strittmatter et al., 1995; Shen et al., 2002). Furthermore, GAP-43

expression is upregulated in regenerating dorsal root ganglion axons and over expression of GAP-43 enhances the degree of regeneration (Bomze, 2001; Gonzalez-Hernandez and Rustioni, 1999). MAPK activity is required for neurite outgrowth in PC12 neurons (Kolkova et al., 2000; Walowitz and Roth, 1999), mouse dorsal root ganglion axons (Sjogreen et al., 2000), mouse cerebellar neurons (Schmid et al., 2000; 1999), and chick retinal neurons (Dimitropoulou and Bixby, 00), but stimulation of MAPKs alone is not sufficient to induce neurite outgrowth.

GAP-43 and MAPKs affect neurite outgrowth in different ways. Whereas GAP-43 is thought to enhance neurite outgrowth by increasing F-actin polymerization (He et al., 1997; Dent and Meiri, 1998), MAPKs are believed to enhance neurite outgrowth by increasing the expression of growth promoting proteins like GAP-43 (Yuan et al., 2001; Olsson and Nanberg, 2001; Encinas et al., 1999). Some have suggested that MAPKs act to transform neurites into outgrowth permissive structures by regulating protein expression (Doherty et al., 2000; Olsson and Nanberg, 2001), and that sustained MAPK activity is required for outgrowth to continue in the presence of a stimulatory cue like nerve growth factor (NGF) or nCAM. In fact, some growth inhibiting compounds, like ephrins, act by downregulating MAPK activity (Elowe et al., 2001). Clearly, both GAP-43 and MAPK activity are important for neurite outgrowth, and are required for CAM-mediated outgrowth to occur.

Neurotrophic Factors

Neurotrophic factors are thought to be required for the survival of both developing and adult neurons. Their expression throughout development helps guide neurons to their proper target and their continued expression in the adult is believed to be required for learning and memory (McAllister et al., 1999). Neurotrophic factors act by binding to tyrosine kinase (Trk) receptors, which result in the activation of phospholipase C (PLC) and increased PKC activity (Ming et al., 1999). Other signaling molecules such as ADF, GAP-43, MAPKs and Rho family guanosine 5' triphosphate (GTP)-ases are also involved in mediating their effects on neurite outgrowth.

NGF is among many extracellular signaling molecules that affect the activity of the Rho family GTPases (Ozdinler and Erzurulmu, 2001), which include Rho, Rac, and Cdc42 (for review see Symons and Settleman, 2000). These GTPases are inactive when bound to GDP, but upon binding of GTP, they activate signaling pathways that regulate F-actin polymerization (Bishop and Hall, 2000). Rac and Cdc42 are known to increase rates of outgrowth (Brown et al., 2000), whereas Rho activity significantly reduces outgrowth.

Rho family GTPases control neurite outgrowth through various actions on the actin cytoskeleton. For example, ADF activity is regulated by LIM kinases (Yang, 1998), which are downstream effectors of Rho family GTPases (Kuhn et al., 2000). As previously noted, the activity of ADF can regulate the turnover of F-actin and the rate of neurite outgrowth. Another way that Rho family GTPases affect neurite outgrowth is by directing actin polymerization. Rac and Cdc42 are believed to help form an actin polymerizing complex that contains numerous proteins, and is influenced by multiple tyrosine kinases and protein phosphatases (for review see Korey and Van Vactor, 2000). The involvement of such a wide array of compounds makes this procedure both highly regulated and very sensitive to alterations of the intracellular milieu.

Cytosolic Calcium

Calcium has long been known as a master regulator of cellular functions. Virtually every cell activity is influenced in some way by calcium. Cytosolic calcium regulates the activity of various cellular proteins, including enzymes such as tyrosine kinases and protein phosphatases. Furthermore, calcium itself is tightly regulated, as it is quickly chelated by calcium binding proteins, sequestered into intracellular stores, and pumped out of the cell by calcium ATPases (for review see Pozzan et al., 1994).

In the growth cone, increases in $[Ca^{2+}]_c$ can lead to various different events. As mentioned earlier, submembranous rises in $[Ca^{2+}]_c$ mediate CAM-induced neurite outgrowth, whereas other growth cone calcium transients have been implicated in growth cone turning (Gomez et al., 2001; Zheng et al., 2000), the induction of new filopodia (Lau et al., 1999) and inhibition of neurite outgrowth (Murrain et al., 1990). Although the frequency and amplitude of $[Ca^{2+}]_c$ transients are key factors in determining the response of a growth cone, the resting basal calcium level is also very important. For example, localized increases in $[Ca^{2+}]_c$ induce attractive growth cone turning at normal levels of extracellular calcium, but repulsive turning occurs when basal $[Ca^{2+}]_c$ levels are lowered by the removal of extracellular calcium (Zheng et al., 2000). Clearly calcium is an important signaling molecule, and understanding its regulation of growth cone behavior requires elucidation of its interactions with multiple signaling pathways and cellular domains.

Through the intracellular measurement of $[Ca^{2+}]_c$ with calcium indicator dyes, two types of spontaneous calcium transients have been identified (for review see Gomez and Spitzer, 2000). The first are calcium spikes that consist of large, short lasting increases in $[Ca^{2+}]_c$ (Spitzer , 1994). They result from calcium influx during calcium dependent action potentials and release from intracellular calcium stores (Spitzer, 1994). Blockade of calcium spikes does not influence the rate of neurite outgrowth, but rather suppresses normal phenotypic development of neurotransmitter expression in *Xenopous* spinal neurons (Gomez

and Spitzer, 2000; Spitzer, 1994). Calcium oscillations can increase the efficiency and specificity of gene expression (Dolmetsch et al., 1998), and regulate transcription through their amplitude and duration (Dolmetsch et al., 1997; 1998).

The second type of spontaneous calcium transient is called a wave. It is initiated in the growth cone and spreads via diffusion into the neurite and in some cases all the way to the cell body (Spitzer, 1994). Calcium waves are smaller in amplitude and longer in duration than spikes, and result from extracellular calcium influx that is independent of voltage-gated channels (Spitzer, 1994; Komuro and Rakic, 1996).

Calcium waves occur during neurite outgrowth, and act to regulate the rate of outgrowth. Whereas application of neurotrophic factors to cultured neurons induces both neurite outgrowth and calcium waves (Williams and Cohan, 1994), the waves are not required for neurite outgrowth to occur (Spitzer, 1994). However, the rate of neurite outgrowth is inversely proportional to the frequency of calcium waves (Gomez and Spitzer, 1999), and blocking these waves results in accelerated elongation. Furthermore, the inhibitory effect of calcium waves on neurite outgrowth is mediated by the calcium-dependent phosphatase, calcineurin, which inactivates certain proteins, such as the growth promoting cytoskeletal element GAP-43 (Lautermilch and Spitzer, 2000).

Calcium is a major regulator of F-actin dynamics, one of the key processes underlying neurite outgrowth. Increasing $[Ca^{2+}]_c$ with a calcium ionophore reversibly blocks neurite outgrowth much like a spontaneous calcium wave (Lankford and Letourneau, 1989). This effect is due to the disruption of F-actin and the loss of filopodial bundles (Neely and Gesemann, 1994; Welnhofer et al., 1999). Stabilization of actin blocks the inhibition of neurite outgrowth by calcium (Lankford and Letourneau, 1989).

Evidence from *Helisoma* neurons has demonstrated that elevated $[Ca^{2+}]_c$ affects actin bundle dynamics differently at the proximal and distal ends of filopodia (Welnhofer et al., 1999). Using differential-interference contrast microscopy, filopodial actin bundles were observed to buckle and break upon calcium influx, producing a shortening of the filopodial actin filaments within the lamelipodium. Associated with this response was an inhibition of the retrograde flow of actin filaments and an elongation of the actual filopodia. The data indicate that numerous calcium-induced events are mediated by the activation of calcium-dependent protein kinases and phosphatases (Polak et al., 1991).

The Calcium Hypothesis

Taking into account the wide array of effectors downstream of calcium, it is not surprising that calcium's effect on neurite outgrowth is dependent on many factors, including basal $[Ca^{2+}]_c$, channel expression and modulation, intracellular

stores, chelating and binding proteins, second messenger systems, and even cell size. However, despite all of this complexity, a general hypothesis has been proposed, stating "if intracellular calcium concentration falls below an optimal level, or rises significantly above it, growth cone motility and neurite outgrowth are inhibited" (Kater et al., 1988). An optimal level is present in all growth cones, yet it varies between different neurons (Torreano and Cohan, 1997). Furthermore, the intracellular buffering abilities of cells are important in determining the response to an increase in $[Ca^{2+}]_c$ (Torreano and Cohan, 1997), yet they also differ between cells. For example, a comparison of $[Ca^{2+}]_c$ changes in two different *Helisoma* neurons, B19 and B4, demonstrated different sensitivities of neurite outgrowth to calcium. Whereas neurite outgrowth in neuron B19 was sensitive to small changes in $[Ca^{2+}]_c$, B4 was capable of outgrowth after relatively large increases in $[Ca^{2+}]_c$.

Calcium is proving to be a regulator of neurite outgrowth throughout many different organisms and cell types. Increasing $[Ca^{2+}]_c$ causes a reversible inhibition of neurite outgrowth in *Helisoma* neurons, hippocampal neurons (Song et al., 1994), locus coeruleus neurons (Moorman and Hume, 1994), dorsal root ganglia neurons (Fields et al., 1993; Lankford and Letourneau, 1991), rat sensory neurons (al-Mohanna et al., 1992), and neuroblastoma cells (Silver et al., 1989). This inhibition is mediated by the actions of various compounds, including the calcium binding protein calmodulin (Polak et al., 1991), the calcium-dependent

protein phosphatase calcineurin (Lautermilch and Spitzer, 2000), and the calcium-dependent proteinase calpain (Song et al., 1994).

Since increasing $[Ca^{2+}]_c$ does not inhibit neurite outgrowth in all cell types, the universality of the calcium hypothesis remains in question. For example, in chick dorsal root ganglion growth cones, increasing $[Ca^{2+}]_c$ has no effect on growth cone morphology. However, filopodial length is still regulated by PKC in these cells (Bonsall and Rehder, 1999). Furthermore, in rat sympathetic neurons, increasing $[Ca^{2+}]_c$ by up to 500 nM has no effect on neurite outgrowth (Garyantes and Regehr, 1992). Clearly $[Ca^{2+}]_c$ is a major signaling molecule for the regulation of neurite outgrowth, but its effect is dependent on downstream signaling molecules like calmodulin, calcineurin, and calpains (Gomez and Spitzer, 2000). In cells that are unresponsive to experimentally induced increases in $[Ca^{2+}]_c$, these mediators of calcium function probably carry out different roles than in the majority of cells that adhere to the calcium hypothesis of neurite outgrowth.

Nitric oxide as a regulator of neurite outgrowth

Nitric oxide is a novel neurotransmitter that does not have a typical membrane bound receptor. It is produced in cells by the conversion of L-arginine into citrulline and NO by the enzyme nitric oxide synthase (NOS), of which there are three types: endothelial (e) NOS, inducible (i) NOS, and neuronal (n) NOS.

NO was first identified as the endothelium-derived relaxing factor because it induces vasorelaxation in the cardiovascular system (Johns, 1991). Since then, NO has been implicated in many physiological processes, including neural development and plasticity.

NO is produced throughout development and has been implicated in neuronal differentiation of *Drosophila* (Enikolopov et al., 1999), axogenesis of grasshopper pioneer neurons (Seidel and Bicker, 2000), synaptic refinement of retinal cells (Devadas et al., 2001; Cogen and Cohen-Corey, 2000), and proliferation of Xenopous, PC12 and *Drosophila* cells (Peunova et al., 2001; Enikolopov et al., 1999;). Furthermore, NO has been found in human spinal neurons as they migrate in early development (Foster and Phelps, 2000), but the exact role that it plays is unknown. NO has been found to enhance or inhibit neurite outgrowth depending on the system studied (Hindley et al., 1997; Devadas et al., 2001; Zochodne et al., 1997). For example, increased NOS expression in hypothalamic neurons aids regeneration, whereas NOS activity hinders regeneration of myelinated fibers (Wu and Li, 1993; Zochodne et al., 1997). How does NO induce different effects on the same underlying process in different systems? In fact, NO has many mechanisms of action, and can therefore cause a wide array of effects in different systems and even within a single neuron.

NO acts through ADP ribosylation

NO is a reactive nitrogen species that can covalently attach ADP-ribose onto proteins and alter their function. This has been proposed as a possible mode of action for NO in hippocampal long term potentiation (Schuman et al., 1994). It is possible that this pathway could also be involved in NO's effects on development and neurite outgrowth.

There is some evidence that NO may directly alter the actin cytoskeleton. NO induces ADP-ribosylation of actin in macrophages, affecting pseudopodia formation and substrate adherence (Jun et al., 1996). NO also ribosylates human neutrophil actin, causing alterations in its polymerization (Clancy et al., 1995). These experiments provide a way in which NO could manipulate the reorganization of F-actin in neuronal cells during neurite outgrowth.

NO acts through intracellular calcium stores

Calcium is a known regulator of many cellular functions, and fluctuations in $[Ca^{2+}]_c$ have been found to influence neurite outgrowth and gene expression (Spitzer et al., 1994; Watt et al., 2000). Extracellular calcium is a major source of calcium, but intracellular storage of calcium is proving to be equally important. There are many different types of intracellular calcium stores that are distinct from each other in their location and modes of release (Johnson and Chang,

2000). These stores may be involved in restricting increases in $[Ca^{2+}]_c$ to specific cellular locations and functions. Furthermore, calcium release from intracellular stores helps regulate neurite outgrowth and differentiation (Takei et al., 1998, Hong et al., 2000).

NO has been found to cause the release of calcium from the endoplasmic reticulum (ER), inositol 1,4,5-trisphosphate (IP3)-sensitive stores, ryanodine-sensitive stores, and cyclic ADP-ribose-sensitive stores (Willmott et al., 1995; Volk et al., 1997; Clementi et al., 1996). NO has also been implicated in the refilling of these stores (Ma et al., 1999). Roles for intracellular calcium stores in neurite outgrowth have recently been proposed, including the regulation of growth cone turning (Hong et al., 2000), elongation (Takei et al., 1998), and collapse (Bandtlow et al., 1993). Together these studies suggest that NO may influence neurite outgrowth through intracellular calcium stores, however this hypothesis remains to be tested.

NO acts through the production of reactive nitrogen species

Once NO is produced, it is quickly broken down into the reactive nitrogen species nitrosyl, nitrosonium, and peroxynitrite (for review see Patel et al., 1999). These compounds can regulate the activity of proteins through S-nitrosylation and nitration to affect cellular activities, including signal transduction pathways implicated in neurite outgrowth. Whereas nitrosonium cations

commonly nitrosylate free thiol groups to give S-nitrosothiols, peroxynitrite anions induce the nitration of free tyrosine residues (for review see Hughes, 1999).

Peroxynitrite and nitrosonium have been shown to increase filopodial outgrowth (Cheung et al., 2000), and may do this via activation of PKC (Bapat et al., 2001). Both NO and peroxynitrite are known to activate the mitogen-activated protein kinase (MAPK) cascade downstream of PKC (Yamazaki et al., 2001; Bapat et al., 2001). Furthermore, activation of extracellular-regulated protein kinase (ERK), a MAPK, mediates NO-induced differentiation of rat PC12 cells (Schonhoff et al., 2001) and neuroblastoma cells (Troller et al., 2001). Stimulation of MAPKs induces cyclic-AMP response element binding protein (CREB) phosphorylation in rat cerebral cells and modulates the expression of genes (Schmid et al., 1999). In fact, NO may influence gene expression in many cell types through this pathway and initiate processes like differentiation, axogenesis and proliferation. It has been suggested that MAPKs may help transform cells into a neurite outgrowth-permissible state (Doherty et al., 2000). Furthermore, activation of this pathway in neurons that are already extending neurites can enhance the rate of outgrowth, and in some cases be required for outgrowth.

Downregulation of the MAPK pathway by neurite outgrowth-inhibitory compounds, like ephrins, causes growth cone collapse and neurite retraction (Elowe et al., 2001). Furthermore, activation of this pathway by neurotrophins

and cell adhesion molecules contributes to their stimulation of neurite outgrowth (Sjogreen et al., 2000; Schmid et al., 2000; Kolkova et al., 2000; Encinas et al., 1999). This stimulation is partly accomplished by increasing the expression of growth promoting proteins like GAP-43 (Olsson and Nanburg, 2001; Encinas et al., 1999). Activation of this pathway by NO is probably involved in mediating NO's effects on neurite outgrowth and development. Furthermore, the activation of PKC by NO will also have direct effects on actin turnover, microtubule advance and GAP-43 phosphorylation. However, the contribution of these pathways in response to NO remains to be determined, and likely varies a great deal between cell types.

NO acts through guanylyl cyclase

The most well characterized action of NO is the activation of the enzyme soluble guanylyl cyclase, which catalyzes the production of 3', 5'-cyclic guanosine monophosphate (cGMP). Many of NO's effects are mediated by increases in cGMP, including developmental responses such as axonogenesis in grasshopper pioneer neurons (Seidel and Bicker, 2000), and enhanced neurite outgrowth in PC12 cells and hippocampal neurons (Hindley et al., 1997). In insects, the expression of nitric oxide-sensitive guanylyl cyclase is associated with the maturational phase of neuronal development (Truman et al., 1996). Studies on regenerating *Helisoma* neurons B5 and B19 revealed that NO

stimulates cGMP production primarily in B5, and regulates growth cone morphology (Van Wagenen and Rehder, 1999); see below.

The *Helisoma* model of neurite outgrowth

The pond snail *Helisoma trivolvis* has been used as a model system for *in vitro* and i*n vivo* studies on neuronal development for many years. Its relatively simple nervous system contains large, easily identifiable neurons that regenerate in culture and *in situ* and can be easily studied using a variety of techniques, including whole cell patch-clamping and intracellular imaging. *Helisoma* neurons, when isolated and cultured in brain-condition medium, grow and extend neurites over the substrate (Wong, et al.,1984). The growth cones produced are very large and their structural reorganization is more easily studied than vertebrate growth cones. Work on this model has helped expand our understanding of the role of neurotransmitters and calcium regulation in neurite outgrowth and neuronal development (for reviews see Kater and Mills, 1991; Goldberg, 1998).

Serotonin selectively inhibits neurite outgrowth of a sub-population of *Helisoma* neurons. Although actively growing neurites exhibit higher $[Ca^{2+}]_c$ than

their non-growing counterparts, if $[Ca^{2+}]_c$ levels are raised beyond the outgrowth permissible range, then neurite outgrowth stops (Cohan et al., 1987). Application of serotonin to specific *Helisoma* neurons causes an increase in $[Ca^{2+}]_c$ and an inhibition of neurite outgrowth in approximately 50% of adult and embryonic neurons (Goldberg et al., 1991; 1992). These early experiments on *Helisoma* have helped clarify the role of calcium in neurite outgrowth.

In vivo, the inhibitory effect of 5-HT (serotonin) on neurite outgrowth is required for proper regeneration and development. In *Lymnea*, a close relative of *Helisoma*, during regeneration of crushed giant serotonergic cells, serotonin release acts to fine tune axon guidance and neurite branching (Koert et al., 2001). During the development of *Helisoma*, the release of serotonin from a pair of embryonic neurons auto-regulates their own branching and also controls the development of other surrounding cells (Diefenbach et al., 1995; Goldberg and Kater, 1989). Neuron B19 is an example of a cell whose development is regulated by serotonin. Treating developing embryos with 5,7-dihydroxytryptamine, to lower the levels of serotonin, results in alterations in neuronal morphology, neuronal dye coupling, and an increase in electronic coupling of neuron B19 (Goldberg and Kater, 1989). Taken together, these results suggest that serotonin regulates the development and regeneration of neurons by controlling the degree of neurite outgrowth and synaptogenesis.

Studies on buccal neuron B19 regenerating in culture have elucidated the mechanisms underlying the serotonin-induced inhibition of neurite outgrowth. Application of serotonin to neuron B19 causes a reversible inhibition of neurite outgrowth (Haydon et al., 1984), in part due to the influx of extracellular calcium, a loss of filopodial F-actin bundles, and a collapse of the growth cone (Cohan et al., 1987; Welnhofer et al., 1999). Furthermore, the response to serotonin in neuron B19 is dependent on the calcium binding protein calmodulin, and blocking its activity prevents serotonin-mediated growth cone collapse in neuron B19 (Polak et al., 1991).

The inhibition of neurite outgrowth mediated by serotonin is also dependent on electrical activity. Serotonin inhibits neurite outgrowth in neuron B19 by initiating a cell depolarization and a subsequent calcium influx through voltage gated channels (Mattson et al., 1988; Price and Goldberg, 1993). The same response is also observed in neurons B5 and B19 after electrical stimulation (Cohan et al., 1987). Furthermore, neurotransmitters that inhibit electrical activity can influence neurite outgrowth by preventing action potentials from occurring. For example, acetylcholine, an inhibitory neurotransmitter, blocks serotonin-mediated inhibition of neurite outgrowth in neuron B19 by hyperpolarizing the cell (McCobb et al., 1988). The decision of a neurite to elongate is dependent on its electrical activity, which is regulated by the expression of membrane channels and receptors, and the presence of extracellular neurotransmitters.

Cyclic-nucleotides also play a role in the serotonin response. Cyclic nucleotide-gated channels are reported in the mollusks *Helisoma*, *Aplysia* and *Pleurobranchaea* as well as in vertebrate retinal and olfactory cells (Kirk et al., 1988; Sudlow and Gillette, 1997; Urbina et al., 1996). The response of neuron B19 to serotonin is mediated by a cyclic nucleotide-gated sodium current that depolarizes the membrane and opens voltage gated calcium channels (Price and Goldberg, 1993), causing an inhibition of neurite outgrowth (Mattson et al., 1988). Serotonin may accomplish this by binding to G-protein coupled receptors that activate adenylyl cyclase and increase cAMP levels. Pharmacological analysis of the serotonin response (Price and Goldberg, 1993; Price, 1994), together with the structure of two recently cloned serotonin receptors from *Helisoma* (Mapara et al., 2001), suggest that these receptors are different than vertebrate serotonin receptors that couple to adenylate cyclase. Cyclic nucleotide gated sodium channels are also found in neurons like B5 that do not respond to 5-HT, but presumably to other agents that increase levels of cyclic nucleotides. Therefore, in *Helisoma* there is a conservation of underlying second messenger systems combined with differential expression of membrane receptors.

Serotonin depolarizes neuron B19 by increasing the concentration of cyclic nucleotides within the cell (Price and Golderg, 1993). However, it is not known whether cAMP or cGMP is produced, because both can activate the cyclic nucleotide gated channels that produce the depolarization (Price, 1994). Nitric

oxide, on the other hand, is known to mediate an influx of calcium into neuron B5 through a cGMP-dependent mechanism (Van Wagenen and Rehder, 1999). These results suggests that NO and serotonin both induce an increase in cyclic nucleotides and regulate the electrical activity of *Helisoma* neurons.

NO affects growth cone morphology in neuron B5, but not in neuron B19. However, its role in neurite outgrowth is unknown. Application of NO to neuron B5 causes a cGMP-dependent rise in $[Ca^{2+}]_c$ in growth cones and alterations of their actin cytoskeleton (Van Wagenen and Rehder, 1999). Since NO produced a reduction in filopodial number but an increase in filopodial length, it was suggested that NO transforms the growth cone into a long-range sensor (Van Wagenen and Rehder, 1999). This filopodial response, however, is also produced by experimentally-induced increases in $[Ca^{2+}]_c$ that inhibit growth cone motility (Welnhofer et al., 1999) and neurite outgrowth (Mattson et al., 1988). Therefore, to clarify these contradictory outcomes, the effect of NO on neurite outgrowth must be evaluated in *Helisoma* neurons B5 and B19.

The goal of this study was to determine what affect NO has on neurite outgrowth in *Helisoma* neurons. We decided to measure the effect of NO on the neurite outgrowth of neurons B19 and B5. Neuron B5 is known to contain NO sensitive guanylyl cyclase, yet neuron B19 does not (Van Wagenen and Rehder, 2001). Furthermore, only neuron B5 responds to NO with an increase in filopodial length (Van Wagenen and Rehder, 2001). These results suggest that

NO should differentially affect neurite outgrowth in these cells. Because the expression of guanylyl cyclase appears to differ between neurons B5 and B19, we also wanted to investigate whether NO's effects on neurite outgrowth were due to cGMP. Furthermore, we measured changes in $[Ca^{2+}]_c$ because, as mentioned above, calcium is a strict regulator of neurite outgrowth. This study tests the hypothesis that NO has no effect on neuron B19, whereas it increases cGMP levels in neuron B5 and inhibits neurite outgrowth in a calcium-dependent manner.

Methods:

Animals:

Inbred, albino *Helisoma trivolvis* snails were raised in glass aquaria with oyster-shell substrata. Snails were fed trout chow and lettuce, and were maintained at 25° C on a 12:12 hr light:dark cycle. Adult snails with a shell diameter of 10-18 mm were used for dissection.

NADPH-diaphorase histochemistry:

Whole buccal ganglia were removed from snails, pinned in silicone rubber-coated wells, and fixed at room temperature with 4% paraformaldehyde in *Helisoma* saline (51.3 mM NaCl, 1.7 mM KCl, 4.1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 5.0 mM Hepes) for 1 hr. Ganglia were then rinsed twice with saline, and transferred to a solution of 0.625% βNADPH-tetrasodium salt and 0.125% p-nitroblue tetrazolium salt in 50 mM tris and 0.3% Triton-X100 for 45 min at 37^0 C in the dark. The staining reaction was stopped by washing with cold saline. Ganglia were dehydrated with ethanol (2x 70%, 85%, 95%, and 100%), cleared with methyl salicylate and mounted on glass slides. Ganglia were then viewed through a 20X objective and phase-contrast optics of a Nikon Diaphot microscope and photographed with a 35 mm camera (Nikon).

Cell culture:

Procedures for dissections, buccal ganglia removal, and cell culture have been previously described (Haydon et al., 1985). Snails were anesthetized in

20% Listerine for 20 min and pinned onto a dissecting tray bathed in saline containing 5% gentamycin. Whole buccal ganglia were removed with buccal-salivary gland connectives intact. Ganglia were then treated with 0.2% trypsin for 15 min and rinsed in 2 ml of *Helisoma*-defined medium (50% Liebovitz-15, Gibco, Grand Island NY, 40.0 mM NaCl, 1.7 mM KCl, 4.1 mM $CaCl_2$, 1.5 mM $MgCl_2$, 5.0 mM HEPES, 50 μg per ml gentamycin, 0.015% L-glutamine; pH 7.35-7.4) for 45 min. Ganglia were then pinned securely down on silicone rubber-coated dishes and neurons B5 and B19 were identified. All nerve trunks and the buccal commissure were crushed to sever the axons of B5 and B19. An electrolytically-sharpened tungsten wire was used to pierce the connective sheath, directly adjacent to the identified neuron, without making contact with the cell body. The identified neuron was carefully everted from the ganglion sheath by applying pressure to the sheath on the side of the neuron opposite the incision. In no time during this process was the identified neuron directly contacted. The exposed neuron was then suctioned into a glass micro-pipette that had been fire polished and coated with Sigma-coat (Sigma).

Neurons were plated onto poly-L-lysine-coated (MW 70,000 - 150,000, 0.25 mg/ml; Sigma) 35 mm plastic cell culture dishes (Falcon 3001). For calcium imaging experiments, neurons were plated onto poly-L-lysine coated glass coverslips that were attached with silicone adhesive(GE Canada) to the drilled out bottoms of cell culture dishes (Falcon 1008). Cells were incubated in 2 ml of conditioned medium for 12-48 hr at room temperature in a humidified culture

chamber. Conditioned medium was made by incubating 2 snail "brains" per ml of defined L-15 medium for 72-120 hr, a process that adds outgrowth-promoting trophic factors to the medium (Wonget al., 1981). Calcium free medium contained the concentrations for defined medium described above, except that $MgCl_2$ was substituted for $CaCl_2$.

Neurite outgrowth experiments:

Neurite outgrowth experiments were performed 12-48 hr after cells were plated. Photo-micrographs were taken with a 35 mm camera mounted onto a Nikon Diaphot microscope, and imaged under the 20X objective using phase-contrast optics. Neurites were observed for a 1 hr period of controlled outgrowth before the application of drug. Drugs were applied for 1 hr before being washed out with fresh defined media, except in the case of 8-bromo-cGMP, which was applied for 2 hr. Drugs were applied by a complete media exchange. The amount of neurite outgrowth was measured for each individual neurite from photo-micrographs taken every 20 min. The leading edge of the lamelipodium was used to indicate the neurite terminus at each time point. Neurites that did not grow or those that intersected other neurites were not included in the analysis. Imperfections (scratches) in the culture dish were used as reference points to accurately measure the distance of outgrowth between each 20 min interval. Total elongation distance was then graphed for each individual neurite and a line of best fit was made (regression analysis) for the control, drug and washout phases. The slope of the line represented the rate of outgrowth for that

neurite during that particular 1 hr time period. The slopes were then compared before, during, and after drug application using a Student's paired t-test to determine statistical significance. Cross-treatment differences were determined using a Student's unpaired t-test. Differences were considered statistically significant at $p < 0.05$. The average slope from all neurites for each experimental group was used to determine the average rates of outgrowth. For each experiment, neurites from at least 2 different neurons from 2 different culture dishes were examined.

Calcium Measurements:

Neurons were loaded with Fura 2 by incubating them in defined medium containing 10 μM Fura 2-AM dissolved in DMSO with 20% pluronic acid, for 45 min, at 28^0C, and then rinsed with *Helisoma* saline. The fluorescent signal generated by Fura 2 can be considered a useful estimate of $[Ca^{2+}]_c$ (Grynkiewicz et al., 1985). Little or no compartmentalization of Fura 2 fluorescence was seen under the loading conditions used.

Neurons were alternatively excited with a 100 W Hg-Xe arc lamp (Hamamatsu, Japan) at 340 nm and 380 nm wavelengths using a computer controlled filter wheel (Empix Imaging). Emission fluorescence was measured at 510 nm using an intensified CCD camera and the 40X oil-immersion objective of a Zeiss inverted microscope. Fluorescence measurements were collected using custom software (gift from S.B. Kater). Images were captured every minute to

reduce photobleaching over the course of the experiment. Drugs were applied with a complete medium exchange, except for growth cone imaging experiments, where drugs were applied with a perfusion system (Warner Instrument Corp). The ratio (R) of the fluorescence intensity of the two images at 340 nm and 380 nm was converted to obtain an estimate of the calcium concentration, using the formula $[Ca^{2+}] = K_d [(R-R_{min})/(R_{max}-R)] \times [F_o/F_s]$, according to Grynkiewicz et al. (1985). *In vitro* calibration was done using a calcium calibration buffer kit (Molecular Probes).

Drugs:

S-Nitroso-N-acetyl-D,L-penicillamine (SNAP) was freshly prepared as a 100 mM stock solution in dimethylsulfoxide (DMSO; Sigma) and applied to the dish at a final concentration of 100 μM. SNAP is a NO donating S-Nitrosothiol which breaks down to form NO and the corresponding disulphides (Hughes, 1999). To control for a possible effect of this disulphide, 100 μM SNAP was exhausted by exposure to light for 24 hr. 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ; Sigma), a selective inhibitor of NO-sensitive soluble guanylyl cyclase in both mammals (Garthwaite et al., 1995) and mollusks (Van Wagenen and Rehder, 1999; Koh and Jacklet, 1999), was dissolved in DMSO at a stock concentration of 10 mM and stored at -20^0C. ODQ was added to the dish at a final concentration of 10 μM. 8-Bromo cGMP (Sigma), a membrane permeable analogue of cGMP, was dissolved in distilled water and made freshly before each experiment and added to the dish at a final concentration of 1 mM. All drugs

were made up in 2 ml of L-15 medium. The concentration of DMSO in the dish did not exceed 0.1%.

Results

I) NADPH-diaphorase indicates NOS activity in the buccal ganglia

Staining of the buccal ganglia for NADPH-diaphorse resulted in confirmation of previous findings that B5 contains NOS. B5 was one of the most intensely stained neurons observed in the buccal ganglia (Figure 1). Neuron B19 exhibited weak staining of NADPH-diaphorase (Figure 1). This is contrary to previous findings suggesting an absence of NOS activity in this neuron (Van Wagenen and Rehder, 2001). Furthermore, NADPH-diaphorase staining was observed in other buccal ganglion neurons, as well as in buccal commissural axons (Figure 1).

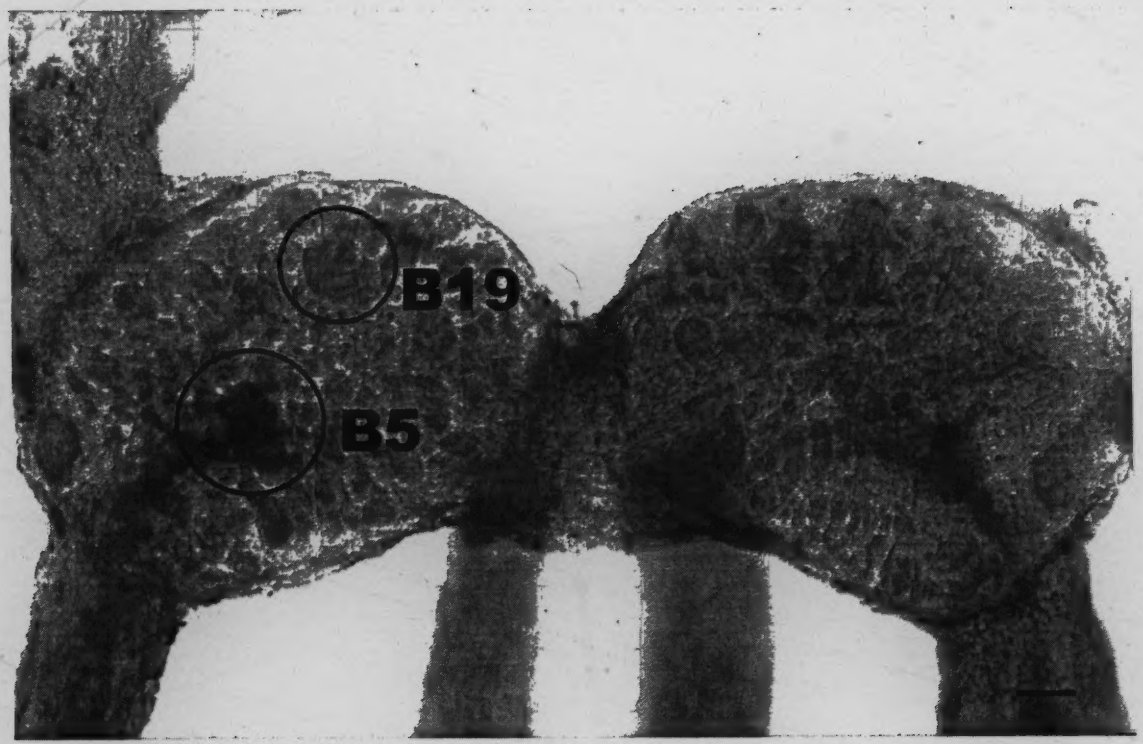

Figure 1: NADPH-diaphorase staining of the buccal ganglion of *Helisoma* reveals many neurons that may contain neuronal nitric oxide synthase. Neurons B19 and B5 are highlighted and are positively stained. (scale bar = 100 um)

II) Rate of neurite outgrowth of neurons B5 and B19

The average rate of outgrowth for neurites extending from neuron B19 was 15.2 ± 1.2 μm/hr. An example of the typical degree of neurite outgrowth observed in neuron B19 after 12-48 hr is demonstrated in Figure 2. Furthermore, this figure also demonstrates the amount of outgrowth observed over the experimental time frame of 2-3 hr. The frequency distribution for the rate of neurite outgrowth in neuron B19 is shown in Figure 3. Most neurites grew at a rate of 10-19 μm/hr, with an overall range between 3 and 28.5 μm/hr.

The average rate of neurite outgrowth observed in B5 neurites was 18.2 ± 0.3 μm/hr, with a range between 7 and 51 μm/hr. An example of the amount of neurite outgrowth observed in neuron B5 is shown in Figure 4. A novel finding was that neuron B5 exhibited 2 populations of growing neurites (Figure 5): a fast population (> 30 μm/hr), and a slow population (< 30 μm/hr). Slow growers advanced at a rate of 15.3 ± 0.7 μm/hr whereas fast growers advanced at a rate of 36.9 ± 1.2 μm/hr. These 2 populations were found on different neurites originating from the same neuron. However, slow growing neurites were around 10 times more prevalent than fast growing neurites.

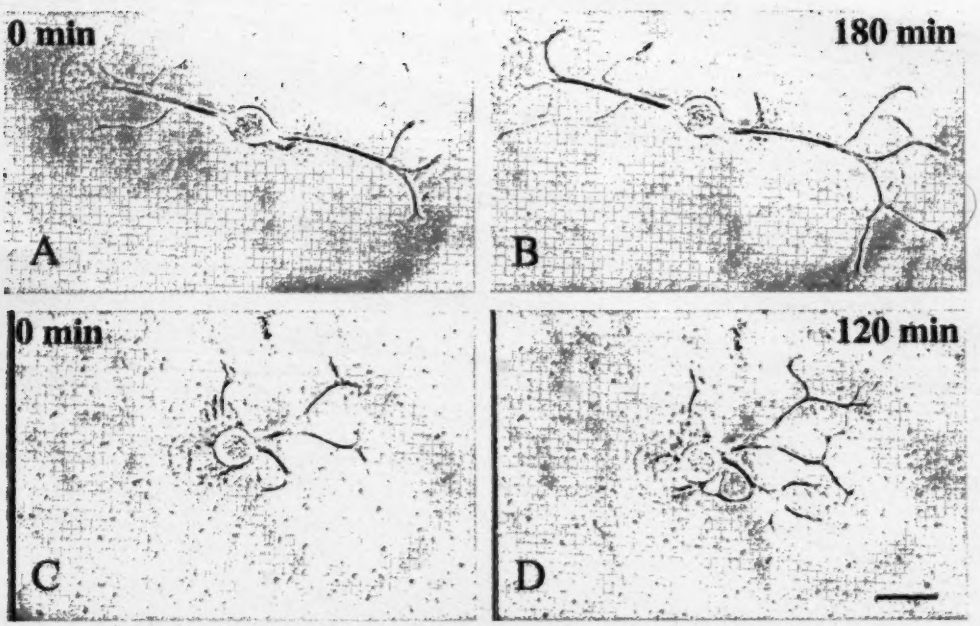

Figure 2: Examples of the typical amount of neurite outgrowth seen in cultured B19 neurons after 12-48 hrs. A+B demonstrate outgrowth in a B19 neuron over 180 min; B+C demonstrate outgrowth in a B19 neuron over 120 min. The marks and lines on the dish were used as reference points when measuring outgrowth. (scale bar = 100 um)

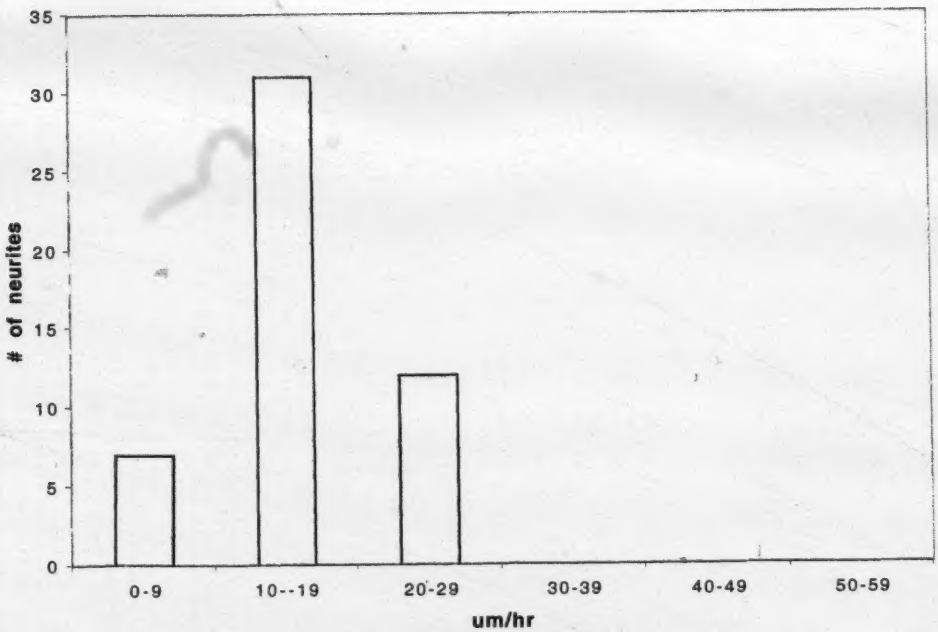

Figure 3: A frequency distribution of rates of neurite outgrowth for neuron B19. Their average rate of outgrowth was 15.2 ± 1.2 um/hr, with a range of 3 to 28.5 um/hr.

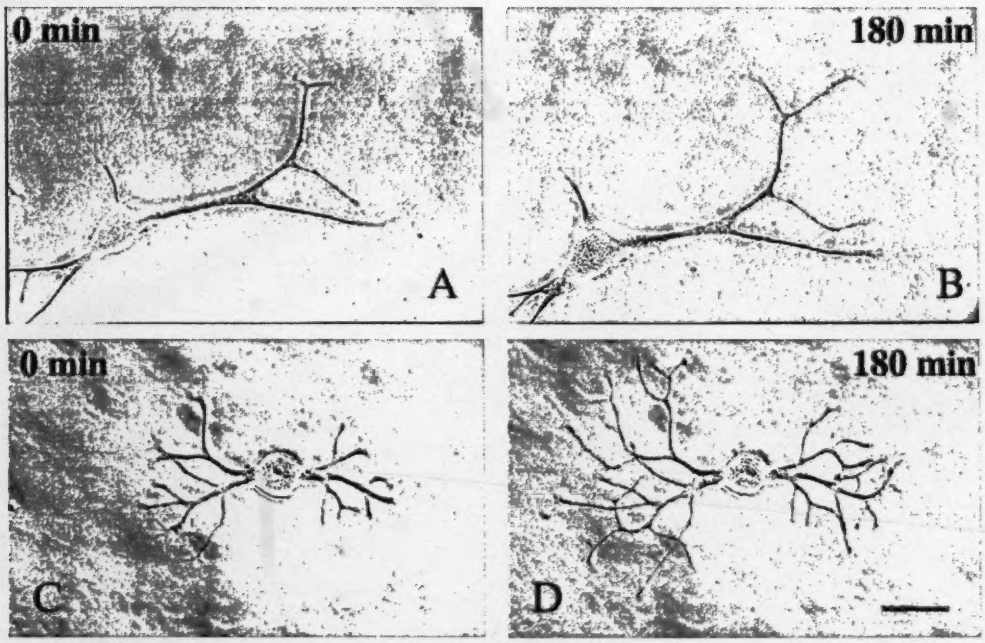

Figure 4: Examples of the amount of outgrowth in cultured B5 neurons after 12-48 hrs. The degree of outgrowth over 180 min is also demonstrated. A+B; C+D demonstrate the distance of neurite outgrowth Over 180 min in 2 different B5 neurons. (scale bar = 100 um)

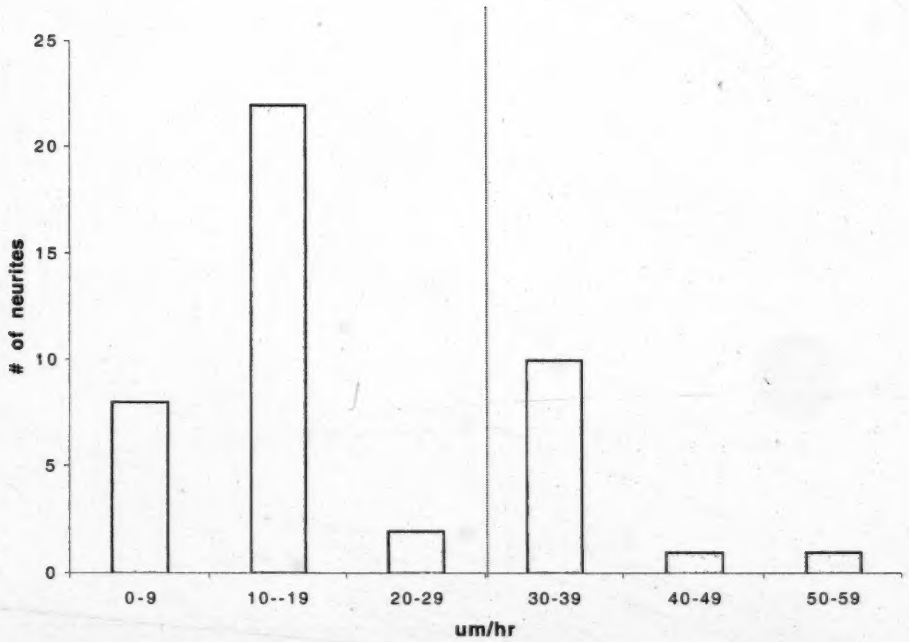

Figure 5: Frequency distribution of rates of neurite outgrowth for neuron B5. This neuron exhibited a bimodal distribution. The neurites were separated into two populations: fast (>30 um/hr) and slow (<30 um/hr). This was done because two different populations seemed to be present, and because these two populations had significantly different responses to the application nitric oxide donor.

III) The effect of NO on neuron B19

We next measured the effect of NO on neurite outgrowth and $[Ca^{2+}]_c$ in neuron B19. Furthermore the role of cyclic nucleotides in mediating the effects of NO was also investigated.

i) NO enhances neurite outgrowth of neuron B19

The application of 100 μM SNAP to neuron B19 caused an increase in the rate of neurite outgrowth to 180.9 ± 12.0 % of control. An example from an individual neurite is demonstrated in Figure 6, where the application of NO donor increased the rate of outgrowth from 7.5 μm/hr to 26 μm/hr. The application of 100 uM SNAP to neuron B19 caused the average rate of neurite outgrowth to increase from 14.2 ± 1.2 μm/hr to 25.7 ± 1.7 μm/hr (n= 23 neurites, 8 cells; $p<0.00005$; Figure 7). The rate of outgrowth recovered to control values after donor was washed out.

A number of controls were done to ensure that the effect of NO donor was due to the release of NO. The application of exhausted SNAP, which does not produce NO, did not significantly change the rate of neurite outgrowth in neuron B19 (n= 8 neurites, 3 cells; Figure 8). To account for the possible involvement of DMSO in the observed response to SNAP, a solution of 0.1 % DMSO was added to neuron B19. This solution did not significantly affect the rate of neurite

outgrowth (n= 7 neurites, 2 cells; Figure 9). Therefore, the observed increase in neurite outgrowth was caused by the release of NO from the donor.

ii) Role of Ca2+ in the effects of NO on neurite outgrowth

To assess whether the stimulation of neurite outgrowth by NO may involve Ca^{2+}, we measured $[Ca^{2+}]_c$ before and after application of 100 μM SNAP. The average $[Ca^{2+}]_c$ in the cell body of neuron B19 was 204.5 ± 6.3 nm. Actively growing neurites typically have $[Ca^{2+}]_c$ between 100-300 nm (Kater et al., 1988). Application of 100 μM SNAP caused an average increase in $[Ca^{2+}]_c$ of 39 ± 16.6 nM in the cell body of B19, however this was not significant (n= 5; Figure 10).

We next tested whether the increased rate of outgrowth caused by NO was dependent on extracellular calcium. Application of 100 μM SNAP to neuron B19 in calcium-free medium caused an increase in neurite outgrowth comparable to that observed in calcium-containing medium (n= 13 neurites, 4 cells; $p< 0.0001$; Figure 11). Furthermore, application of NO donor in the presence of 50 μM $CdCl_2$, a calcium channel blocker, also caused a similar increase in neurite outgrowth (n= 6 neurites, 2 cells; $p< 0.01$ Figure 12).

iii) Cyclic GMP does not mediate NO's effect on neurite outgrowth in neuron B19

The cyclic nucleotide cGMP is known to mediate many effects of NO. Cyclic GMP is also known to increase $[Ca^{2+}]_c$ in neurons B5 and B19 (Van Wagenen and Rehder, 1999; Price, 1994). Therefore, we wanted to determine if cGMP mediates NO's effect on neurite outgrowth in neuron B19.

ODQ, an inhibitor of soluble guanylyl cyclase (Garthwaite et al., 1995; Koh and Jacklet, 1999), was applied to neuron B19 5 min before application of NO donor. The concentration of ODQ used has previously been found to inhibit gyanylyl cyclase in *Helisoma* neurons (Van Wagenen and Rehder, 1999). Application of 100 μM SNAP after ODQ treatment caused an increase in neurite outgrowth similar to NO donor alone ($n=10$ neurites, 3 cells; $p<0.01$). This suggests that NO increases neurite outgrowth independently of cGMP production

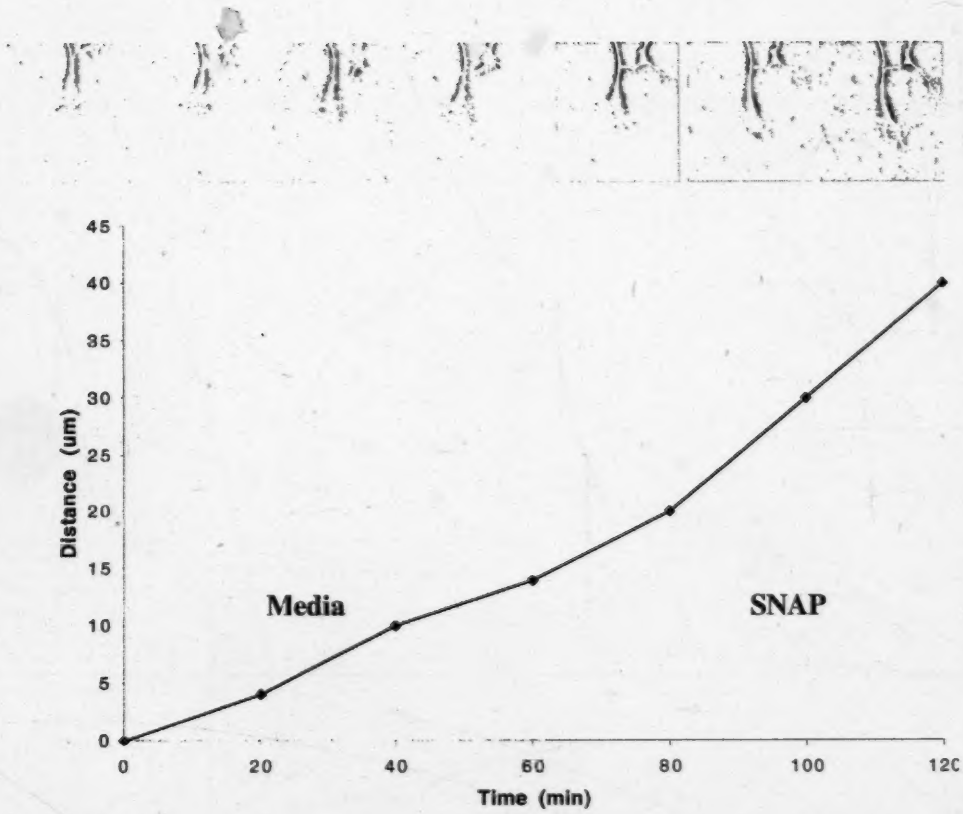

Figure 6: NO donor enhances neurite outgrowth in an individual B19 neurite. The top portion of this figure contains photo-micrographs taken every 20 min of an advancing neurite. The graph represents the total elongation over time.

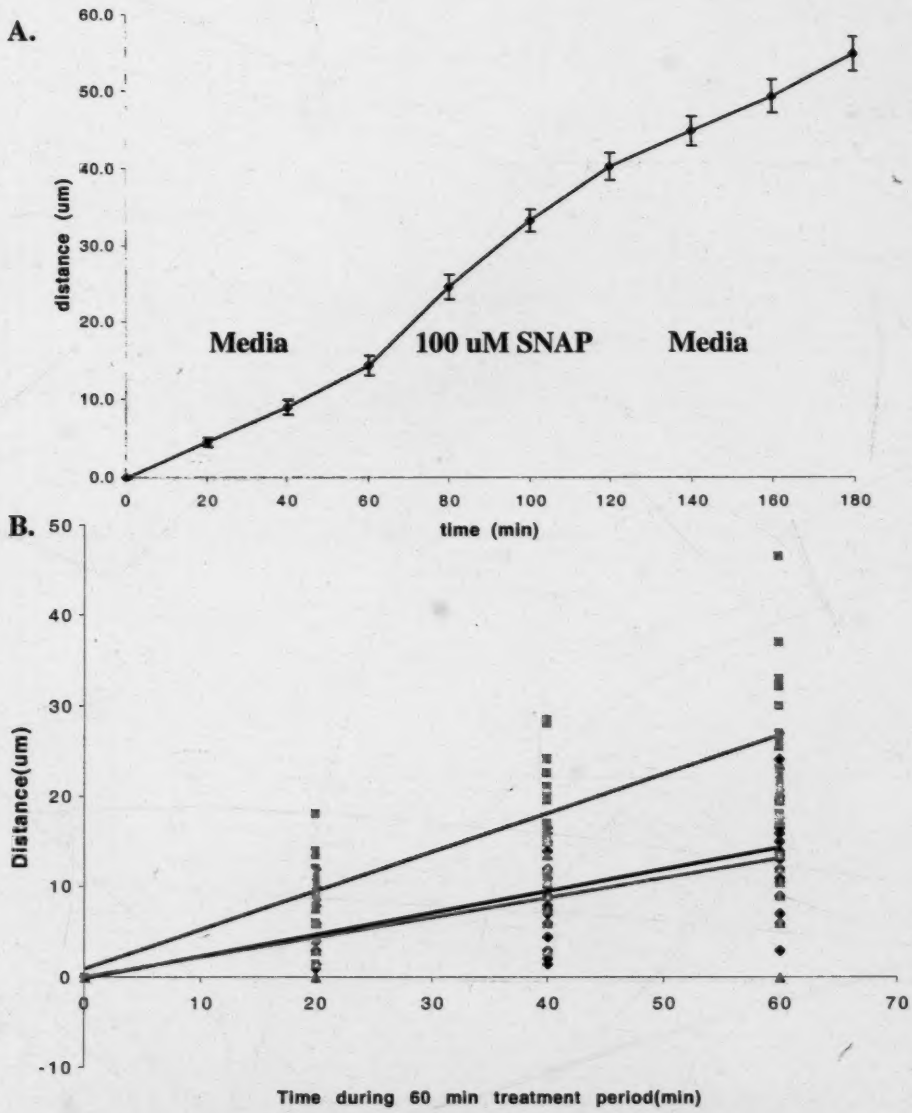

Figure 7: SNAP increased the rate of neurite outgrowth in neuron B19. A: The average distance of neurite outgrowth for neuron B19 over time. B: The average rate of neurite outgrowth for neuron B19 before◆, during■ and after △ application of SNAP, as indicated by the linear regression for each 60 min treatment period. There was no significant difference between the control and wash groups.

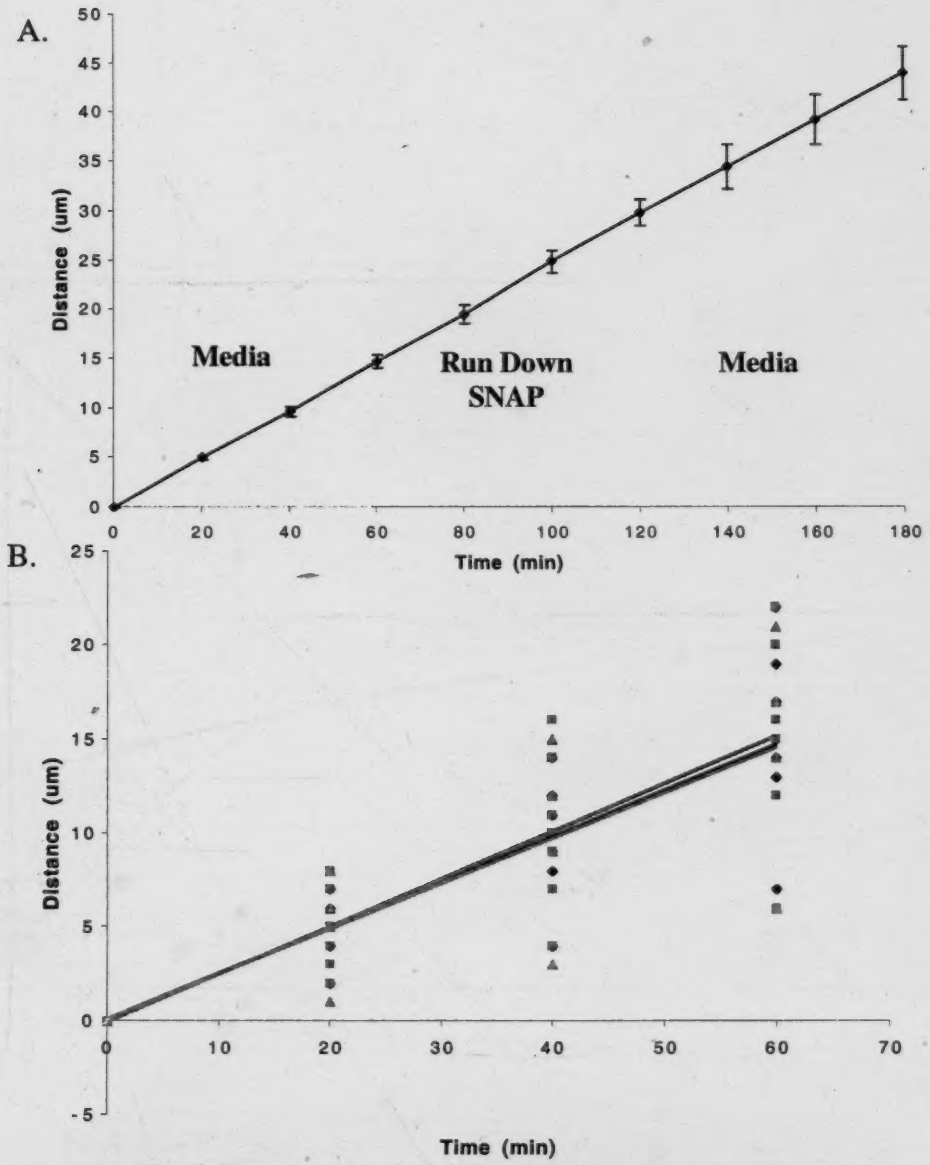

Figure 8: Application of rundown SNAP did not affect the rate of neurite outgrowth in neuron B19. A: The average distance of neurite outgrowth for neuron B19 over time. B: The average rate of neurite outgrowth for neuron B19 before◆, during■ and after △ application of run down SNAP.

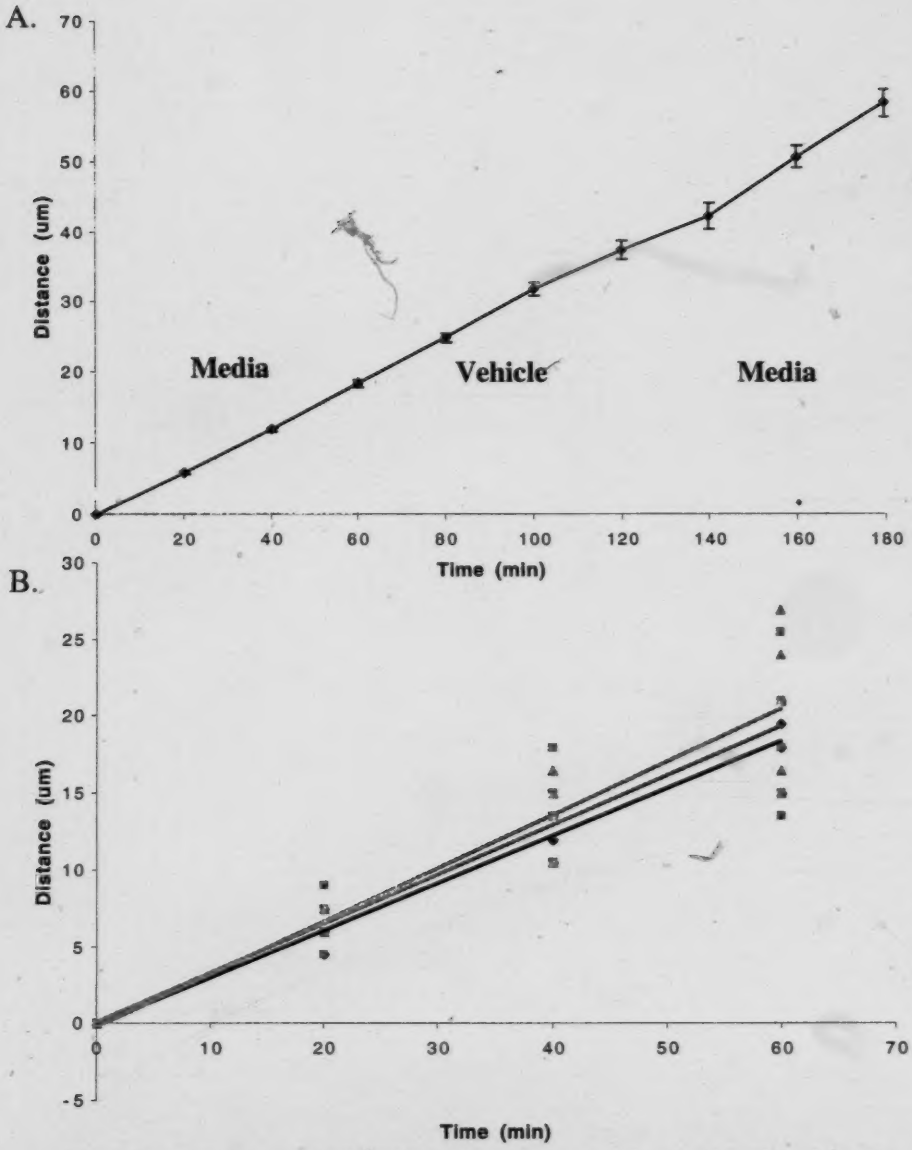

Figure 9: Application of a 0.1% DMSO vehicle did not affect the rate of neurite outgrowth in neuron B19. A: The average distance of neurite outgrowth for neuron B19 over time. B: The average rate of neurite outgrowth for neuron B19 before◆, during■ and after △ application of DMSO vehicle.

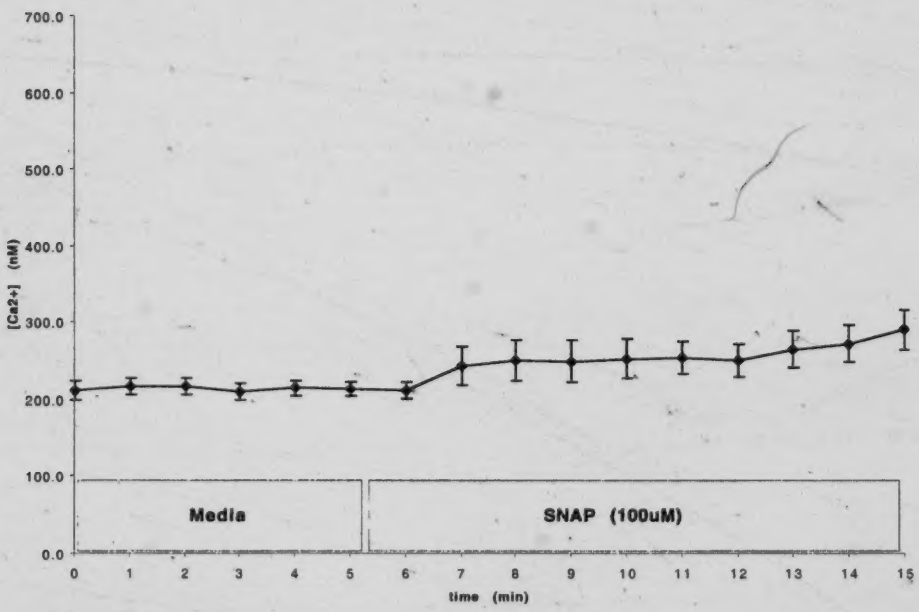

Figure 10: SNAP caused a rise in $[Ca^{2+}]_c$ in neuron B19. The application of nitric oxide donor caused an increase in $[Ca^{2+}]_c$ of 39.7 ± 16.6 nM after 5 min. There was no rise seen until 2 min after application of SNAP.

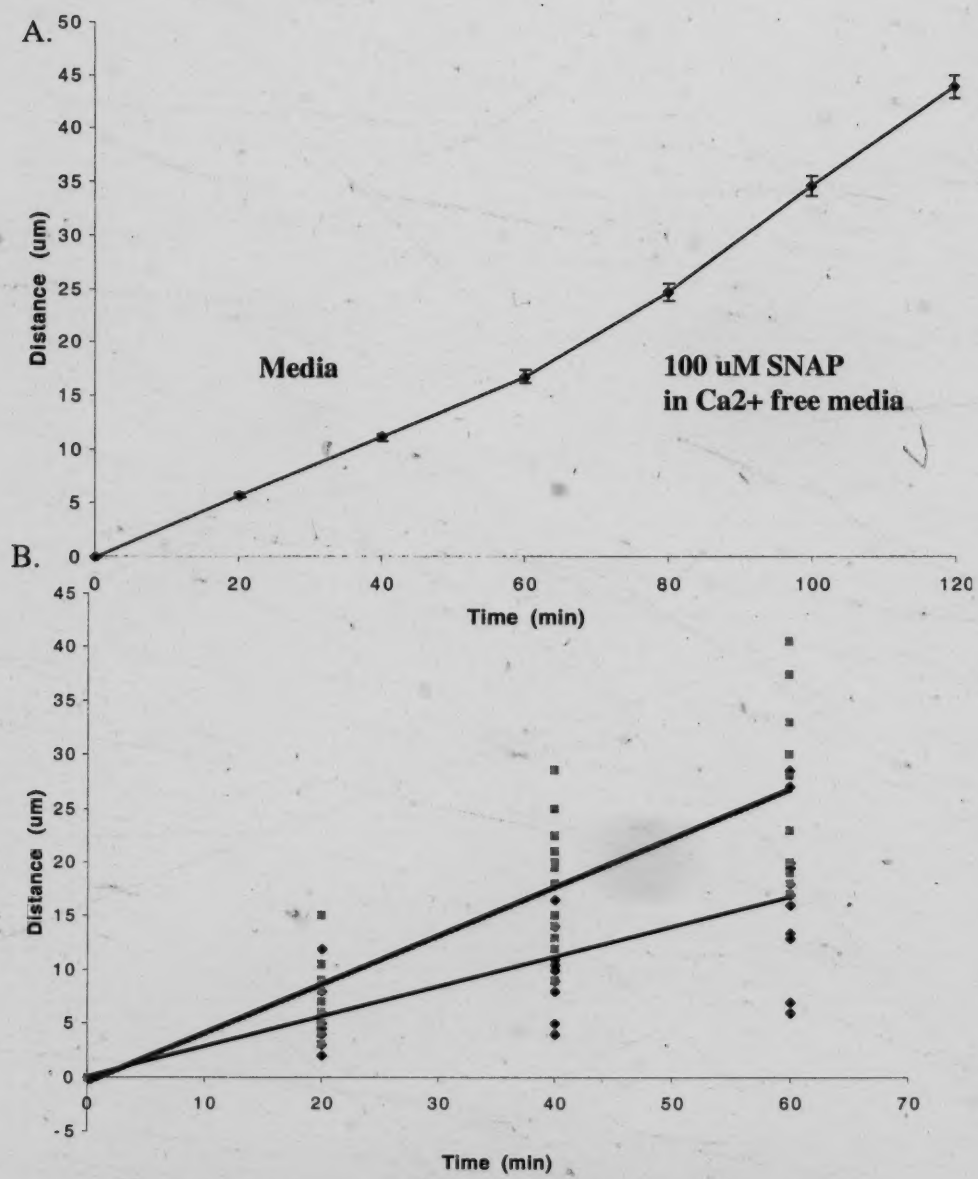

Figure 11: Removing extracellular calcium does not block the effect of SNAP on neurite outgrowth of neuron B19. A: The average distance of neurite outgrowth over time. B: The rate of neurite outgrowth before◆ and after ■ application of 100 uM SNAP in calcium free media.

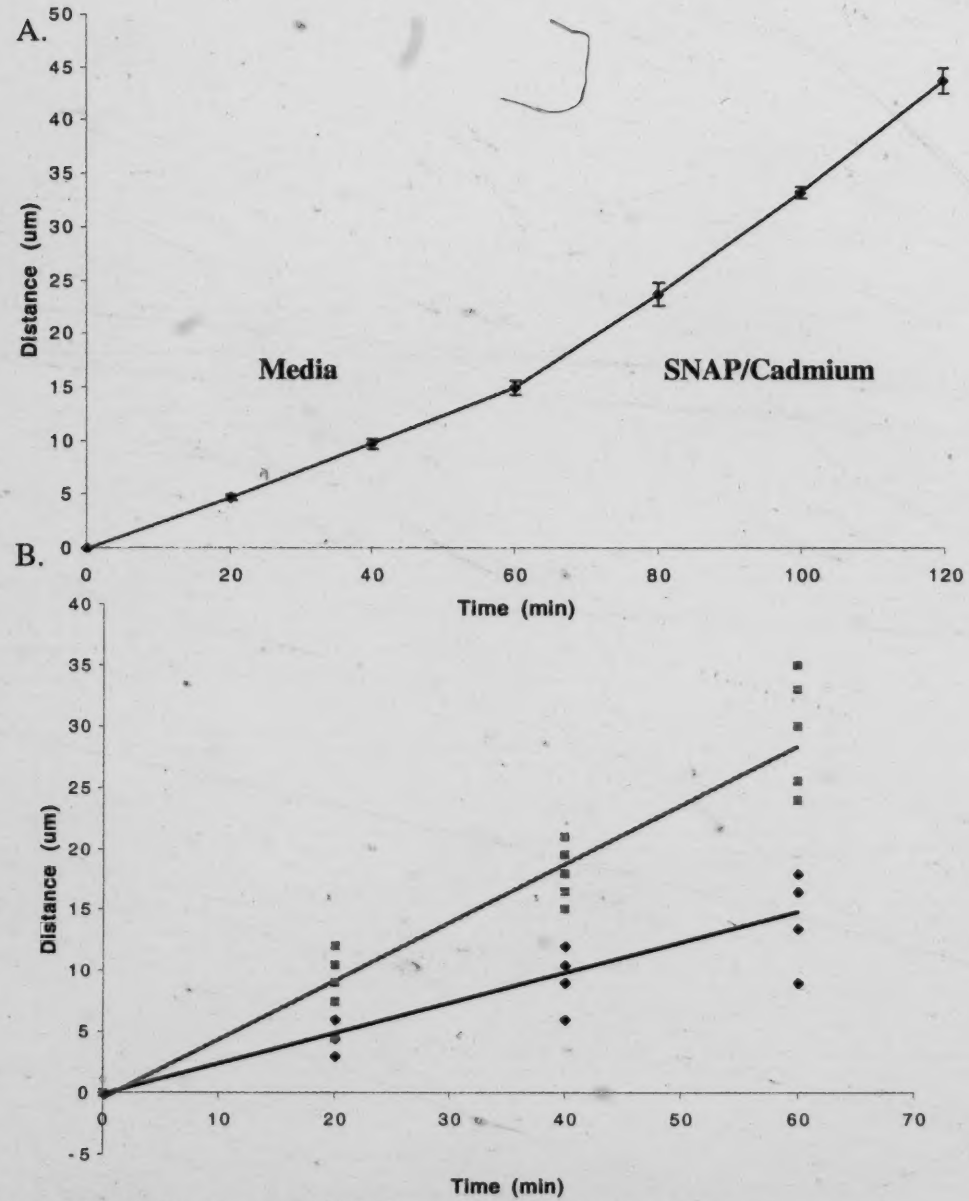

Figure 12: Application of SNAP and the calcium channel blocker cadmium enhances neurite outgrowth. A.) Average distance of neurite outgrowth over time. B.) The rate of neurite outgrowth before ◆ and after ■ application of 100 uM SNAP and 50 uM cadmium.

IV) The effect of NO on neuron B5

Two populations of B5 neurites were uncovered in the frequency distribution analysis of neurite outgrowth (Figure 3). We examined the effect of NO on the rate of neurite outgrowth of each population independently. Furthermore, the roles of extracellular calcium and cGMP were examined in these responses.

i) NO enhances neurite outgrowth in slow growing B5 neurites

Application of 100 μM SNAP to slow growing B5 neurites caused an acceleration of neurite outgrowth (Figure 13) that was significantly smaller to that observed in B19 neurites ($p < 0.05$). The rate of neurite outgrowth increased from and average of 15.3 ± 0.7 um/hr to 21.8 ± 1.6 um/hr (n= 9 neurites , 4 cells; $p < 0.01$; Figure 13) . Upon washout of SNAP, there was only a partial recovery towards control rates of outgrowth.

The application of a DMSO vehicle did not affect the rate of neurite outgrowth (n= 9 neurites, 3 cells; Figure 14). The increase in outgrowth was therefore due to the NO donor, rather than the vehicle.

ii) NO inhibits neurite outgrowth in fast growing B5 neurites

Fast growing B5 neurites did not respond to the application of NO donor in the same way as slow growing B5 neurites. 100 μM SNAP caused an inhibition of outgrowth in fast growing B5 neurites from a rate of 36.9 ± 1.2 μm/hr to 18.1 ± 1.5 μm/hr (n= 7 neurites, 4 cells; $p< 0.02$; Figure 15). This was the only population of neurites to respond to NO with a decrease in the rate of neurite outgrowth.

Fast growing neurites did not recover to control rates of outgrowth after washout of NO donor (Figure 15). Upon washout, neurite outgrowth increased an insignificant amount to 20.4 ± 2.9 μm/hr. Furthermore, after washout of NO, fast growing B5 neurites were advancing at a rate equal to slow growing neurites.

iii) NO increases [Ca2+]c in B5 neurons

Increases in $[Ca^{2+}]_c$ have previously been found to inhibit neurite outgrowth in neurons B5 and B19 (Mattson et al., 1988). Furthermore, the NO mediated increases in $[Ca^{2+}]_c$ in neuron B5 reported by Van Wagenen and Rehder (1999) suggest that NO should mediate a sharp inhibition of neurite outgrowth. However, because we did not observe a sharp NO-mediated inhibition of neurite outgrowth, we wanted to confirm that NO induced an increase in $[Ca^{2+}]_c$ in neuron B5.

Application of 100 μM SNAP to cell bodies of neuron B5 resulted in a sustained elevation of $[Ca^{2+}]_c$, with an average increase of 166.8 ± 39.6 nM measured 5 min after introduction of NO donor (n= 4; Figure 16). This was a much larger increase than that observed in B19 (39 ± 16.6 nM). Because it is possible that an increase in $[Ca^{2+}]_c$ occurred in the cell body but not the growth cones, we decided to directly measure the change in $[Ca^{2+}]_c$ in B5 growth cones. Application of 100 μM SNAP caused an increase in $[Ca^{2+}]_c$ of 154.7 ± 20.5 nM in B5 growth cones measured 5 min after introduction of NO donor (n= 3 growth cones, 2 cells).

iv) Extracellular calcium does not mediate the effects of NO on slow growing B5 neurites

We wanted to determine if the influx of extracellular calcium mediated NO's actions on neurite outgrowth in slow growing B5 neurites. In neuron B5 cultures, calcium-containing medium was replaced by calcium-free medium 5 min prior to the addition of NO donor. This prevented the loss of calcium from intracellular stores, which occurs after prolonged exposure to calcium free medium (Johnson and Chang, 2000). Application of 100 μM SNAP in calcium-free medium caused an increase in neurite outgrowth from 12.4 ± 0.3 μm to 20.6 ± 0.3 μm (n= 10 neurites, 3 cells; $p< 0.0005$; Figure 17.

v) Extracellular calcium inhibits neurite outgrowth in fast growing B5 neurites

Calcium-free medium was also used to determine whether the inhibitory effect of SNAP on fast growing B5 neurites was mediated by a calcium influx. In the presence of calcium-free medium, application of 100 μM SNAP to fast growing B5 neurites had no effect on the rate of neurite outgrowth (n= 3 neurites, 2 cells; Figure 18). This indicates a role for extracellular calcium influx in NO-mediated inhibition of neurite outgrowth.

vi) cGMP does not mediate NO's increase of neurite outgrowth in slow growing B5 neurites

Injection of cGMP into neuron B5 causes an increase in [Ca2+]c similar to the application of 100 μM SNAP (Van Wagenen and Rehder, 1999), and is linked to a change in filopodial activity. To determine if cGMP was mediating the effect of NO on neurite outgrowth in slow growing B5 neurites, we blocked the production of cGMP with a guanylyl cyclase inhibitor 5 min prior to adding NO donor.

In the presence of 10 μM ODQ, application of 100 μM SNAP to slow growing B5 neurites caused an increase in neurite outgrowth greater than that induced by NO donor alone (n= 7 neurites, 2 cells; $p<0.05$; Figure 19). This

provided evidence that cGMP was not involved in accelerating neurite outgrowth, rather it may actually attenuate the increase in neurite outgrowth caused by NO.

vii) cGMP mediates NO's inhibition of neurite outgrowth in fast growing B5 neurites

Since the previous experiments suggested an outgrowth–inhibitory role for cGMP in slow growing B5 neurites, we wanted determine if cGMP mediated the inhibitory effects of NO on fast growing B5 neurites. In the presence of 10 μM ODQ, application of 100 μM SNAP to fast growing B5 neurites did not inhibit neurite outgrowth (n= 4 neurites, 2 cells; Figure 20), and fast growing neurites continued to grow at control rates under these conditions.

To further support cGMP's inhibitory action on neurite outgrowth, we added the membrane permeable analogue of cGMP, 8-bromo-cGMP to slow growing B5 neurites. Application of this analogue (1 mM) has previously been found to take over 1 hr to produce a significant effect on filopodial activity, whereas direct injection of cGMP has an immediate effect (Van Wagenen and Rehder, 1999). Therefore, we examined the effect of 8-bromo-cGMP (1 mM) over a two hour period. Whereas there was no change in the rate of neurite outgrowth during the first hour of incubation, during the second hour there was an inhibition of neurite outgrowth (n= 11 neurites, 4 cells; $p < 0.001$; Figure 21).

This result strengthens our finding that cGMP mediates the NO-induced inhibition of neurite outgrowth in fast growing neurites of neuron B5.

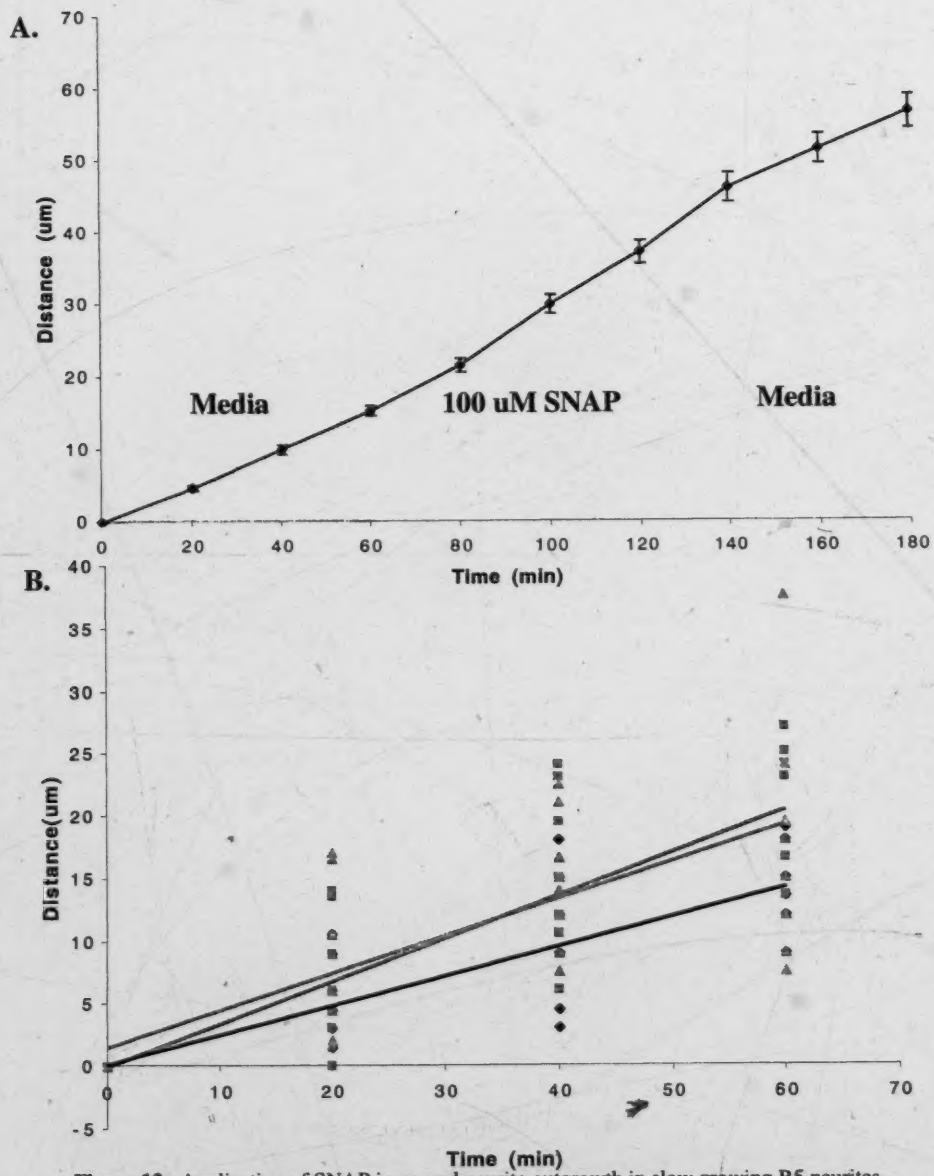

Figure 13: Application of SNAP increased neurite outgrowth in slow growing B5 neurites. A: Average distance of neurite outgrowrth over time. B: The average rates of neurite outgrowth before◆, during■ and after △ application application of SNAP (100 uM SNAP).

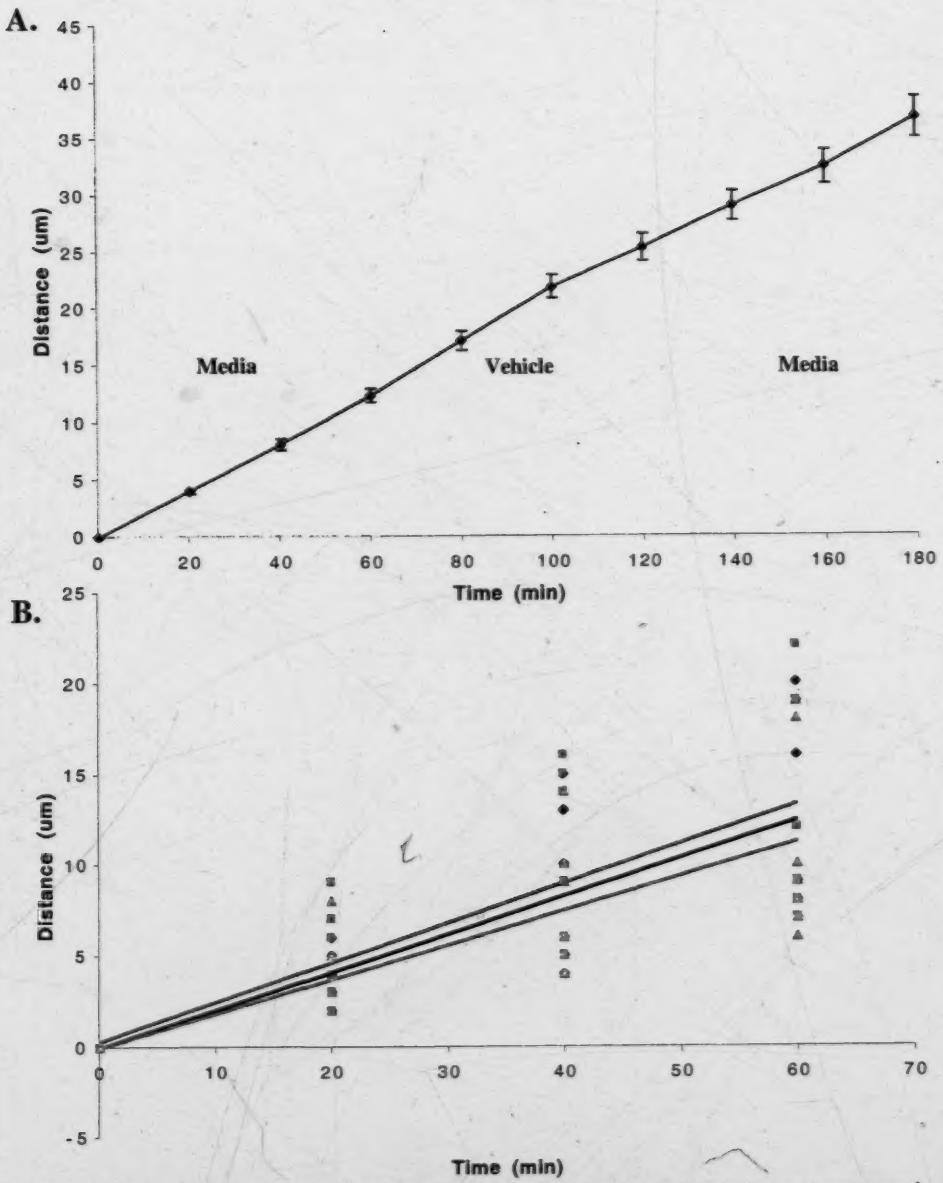

Figure 14: Application of a 0.1% DMSO vehicle caused no significant change in neurite outgrowth in slow growing B5 neurites. A: The average distance of outgrowth over time. B: The average rate of neurite outgrowth before◆, during■ and after △ application of DMSO vehicle.

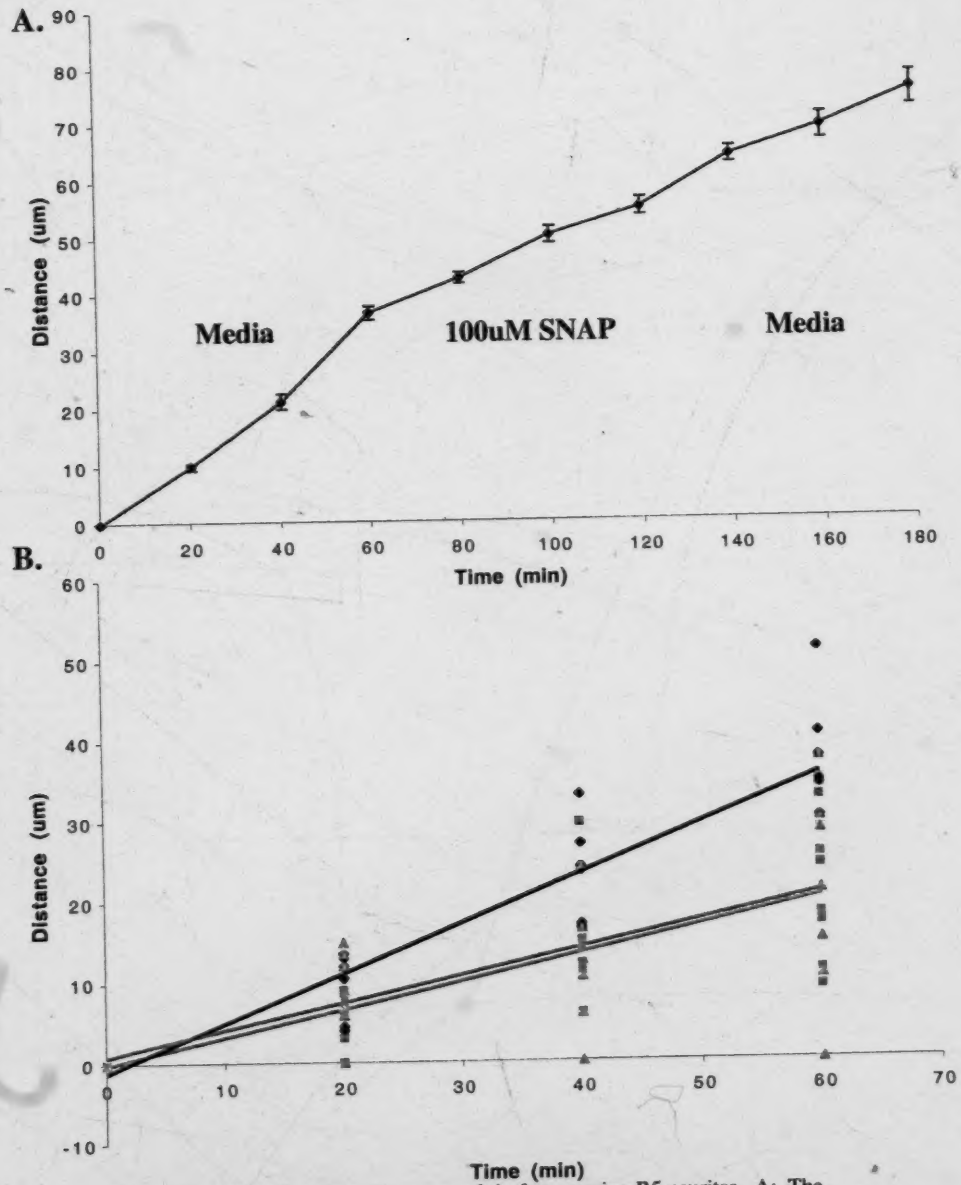

Figure 15: SNAP slows neurite outgrowth in fast growing B5 neurites. A: The distance of neurite outgrowth over time. B: The average rate of neurite outgrowth before◆, during■ and after ▲ application of SNAP (100 uM SNAP).

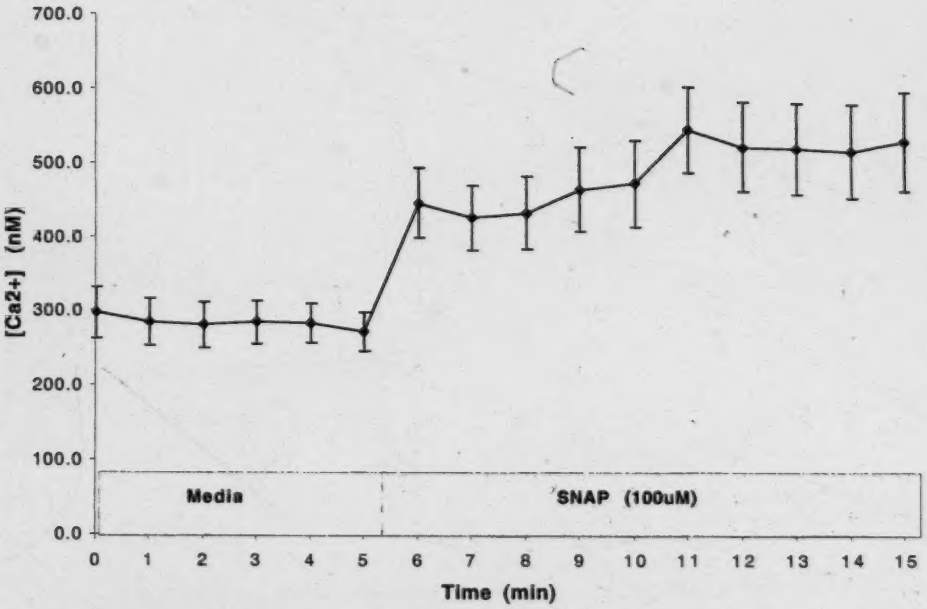

Figure 16: Application of SNAP to neuron B5 caused a large increase in $[Ca^{2+}]_c$ of 166.8 ± 39.6 nM after 5 min.

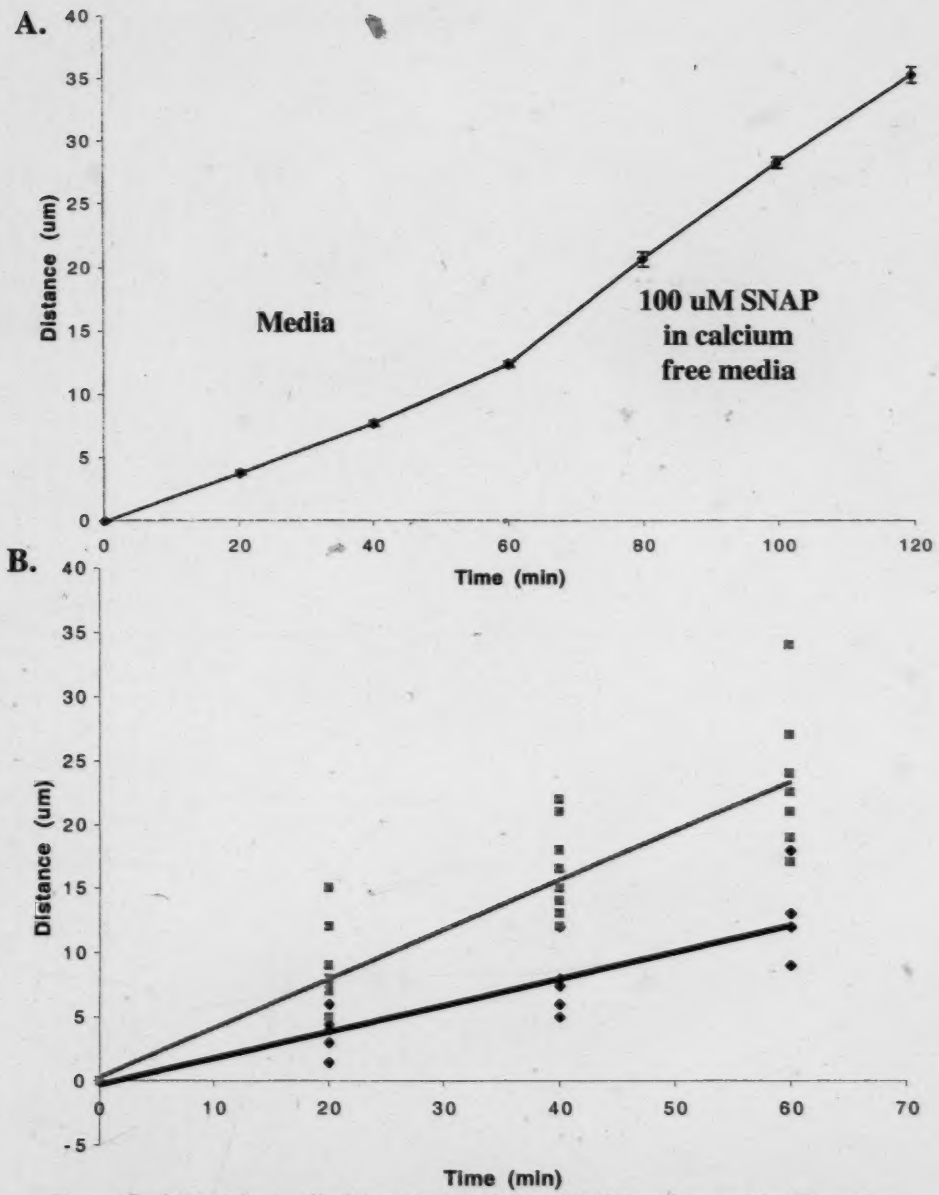

Figure 17: Calcium free media did not block the effect of SNAP on slow growing B5 neurites. A: The average distance of neurite outgrowth over time. B: The average rate of outgrowth before◆, after■ application of SNAP with calcium free media.

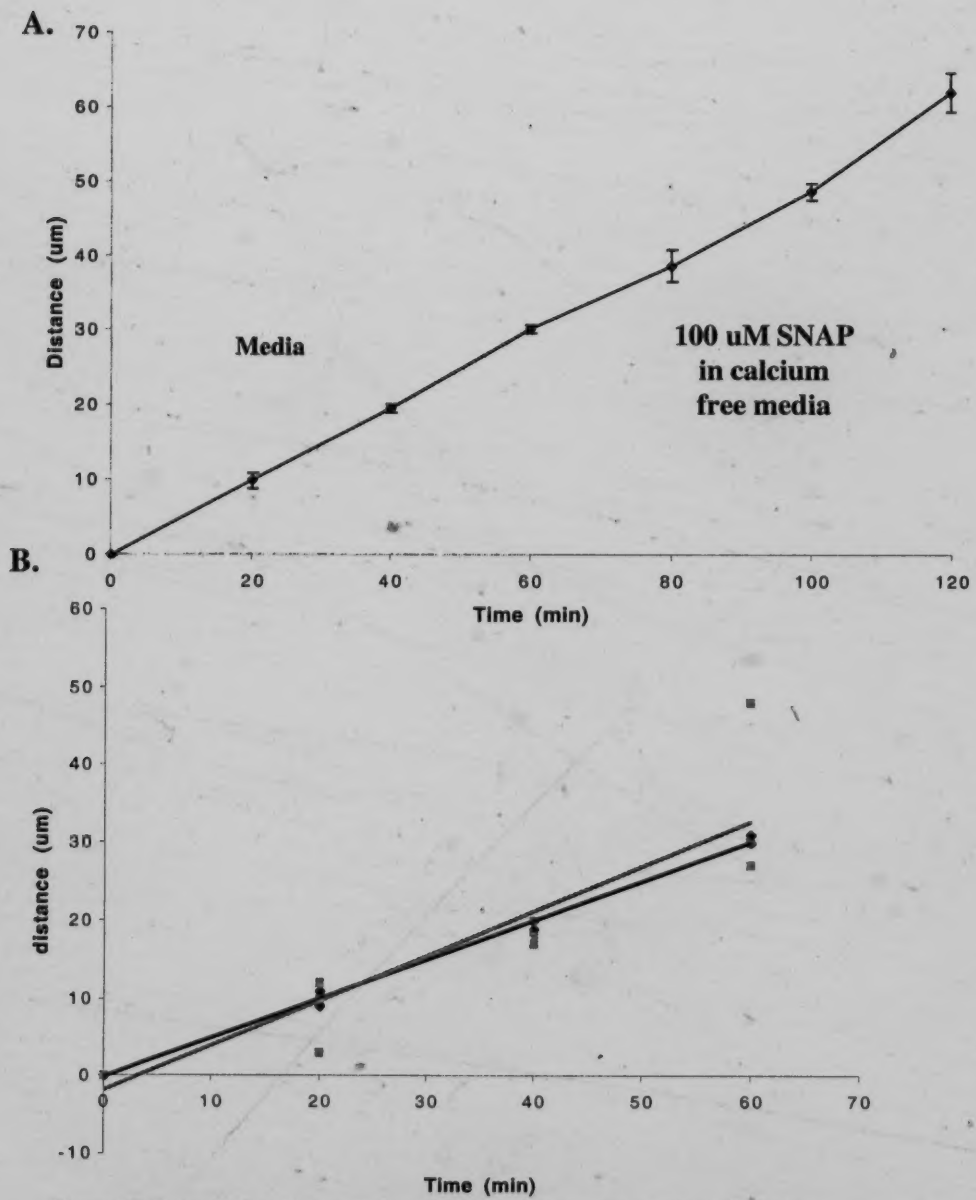

Figure 18: Extracellular calcium mediates SNAP's effect on fast growing B5 neurites.
A: The average distance of neurite outgrowth over time. B: The average rate of outgrowth before◆, after■ application of SNAP with calcium free media.

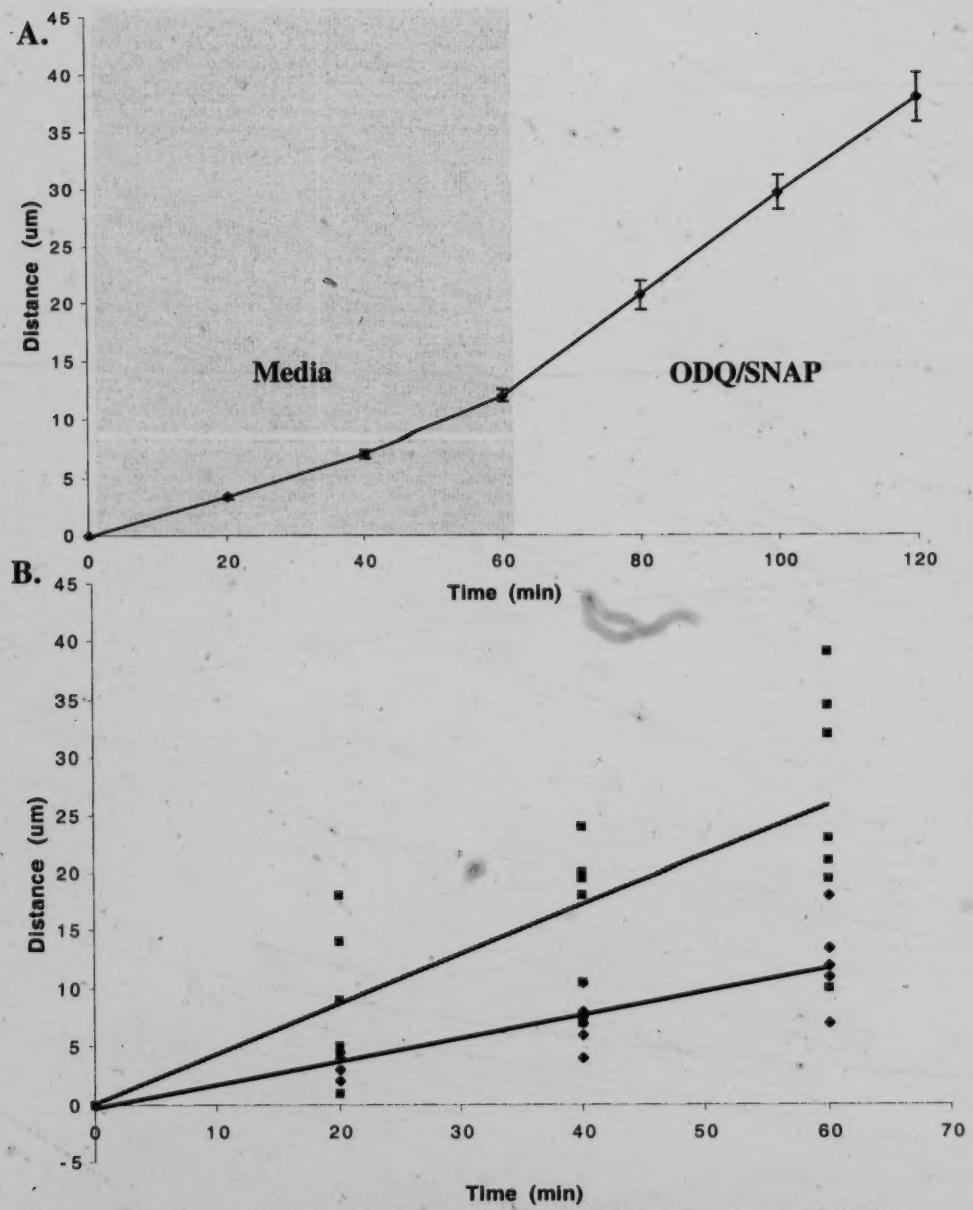

Figure 19: Inhibiting guanylyl cyclase did not block the effect of SNAP on slow growing B5 neurites. A: Average distance of neurite outgrowth over time. B: Average rate of neurite outgrowth of slow growing B5 neurites before◆ and after■ application of 10 uM ODQ and 100 uM SNAP.

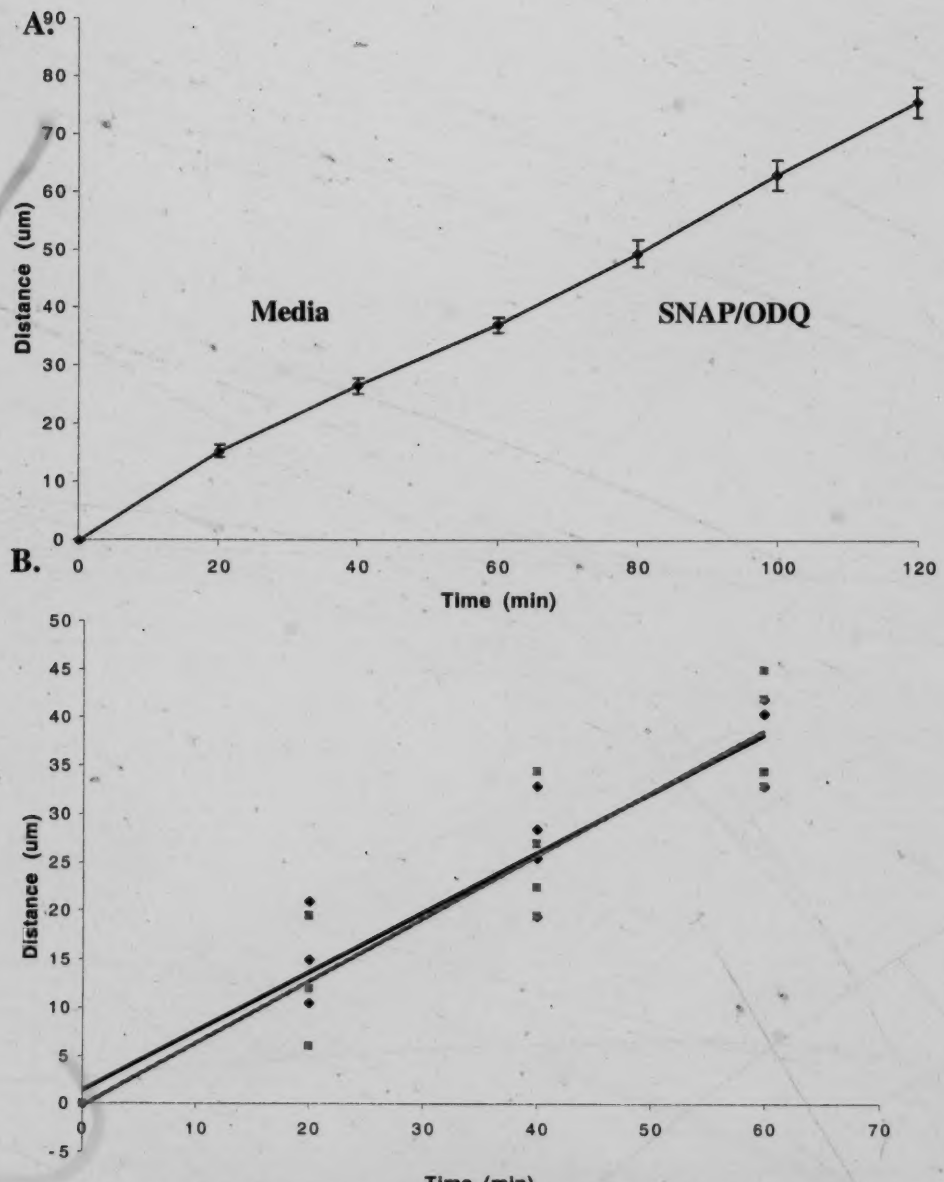

Figure 20: cGMP mediates SNAPs effect on neurite outgrowth of fast growing B5 neurites A: Average distance of neurite outgrowth over time. B: Average rate of neurite outgrowth of fast growing B5 neurites before◆ and after■ application of 10 uM ODQ and 100 uM SNAP.

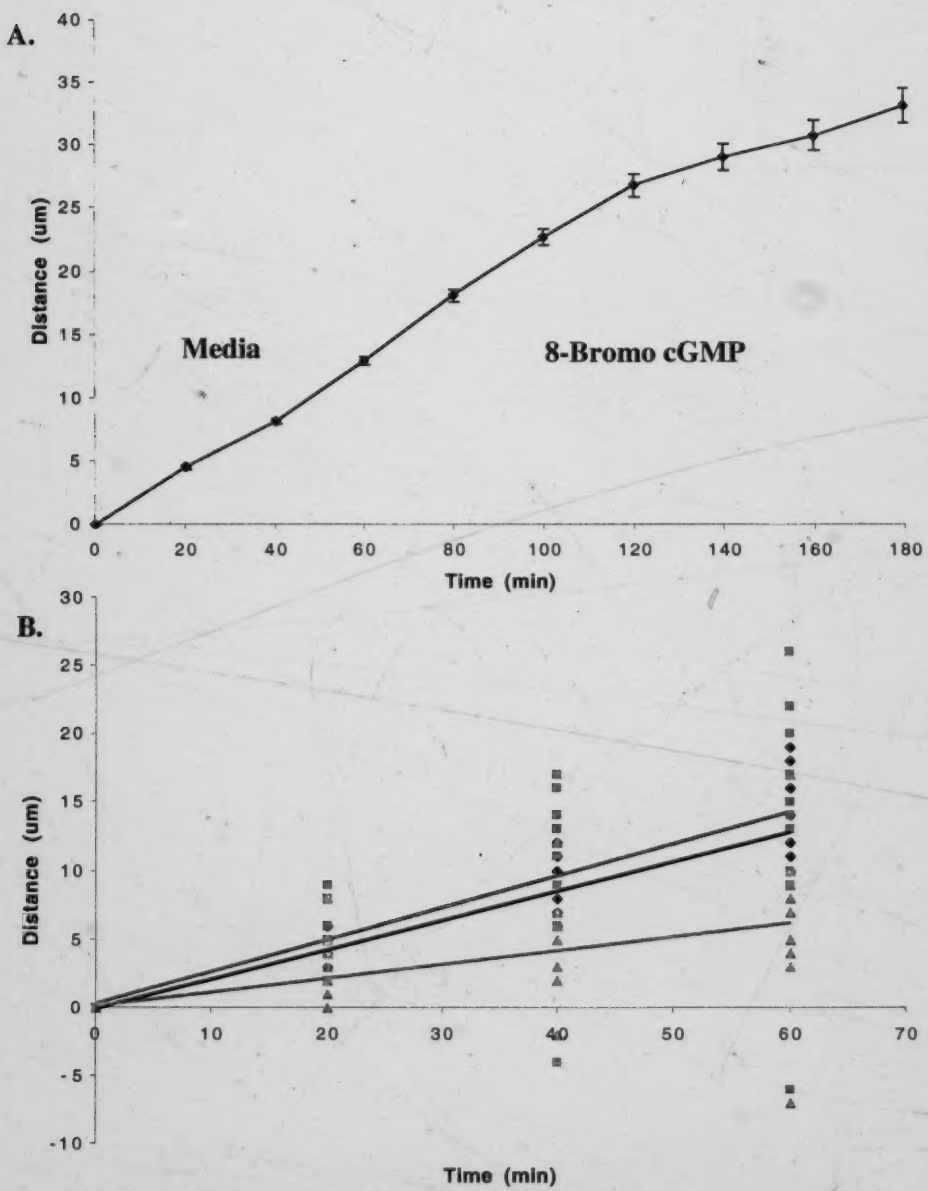

Figure 21: Application of 8-bromo cGMP inhibits neurite outgrowth. A: Average distance of outgrowth over time. B: Average rate of outgrowth of slow growing B5 neurites before◆, 1 hr after■ and 2 hr after △ application of 1 mM 8-bromo cGMP.

Discussion

The experiments outlined above suggest an important role for NO in neurite outgrowth of *Helisoma* neurons. The comparison between neurons B19 and B5 demonstrates physiological differences between neurons within the buccal ganglia, and provides a mechanism for the developmental regulation of neurite outgrowth. Furthermore, there appear to be two separate pathways activated by NO: one independent of cGMP and extracellular calcium influx, and one dependent on both cGMP and extracellular calcium.

NO is produced in buccal neurons

The production of NO is due to the activity of NOS, which can be identified by staining for the co-factor NADPH-diaphorase (Dawson et al., 1991; Hope et al., 1991). We have found that numerous buccal neurons and a large nerve tract running across the buccal commissure stain for NADPH-diaphorase. Positive NADPH-diaphorase staining, as well as NOS antibody staining of buccal ganglion neurons has been observed in other mollusks (for review see Moroz et al., 1996), and represents the importance of NO in molluskan buccal ganglion physiology. NO is produced in the buccal ganglion of numerous mollusks, where it is thought to function in the regulation of the feeding motor program (Elphick et al., 1995). Furthermore, our data demonstrate that NO can regulate neurite

outgrowth, and suggests that it is involved in neuronal regeneration and development.

We have provided evidence that NOS is present in adult neurons B19 and B5, suggesting a physiological role for NO in these neurons. Neuron B5 in particular displayed a large amount of NADPH-diaphorase staining, demonstrating high levels of NOS. This differential expression of NOS may indicate that *in vivo*, NO acts as a neurotransmitter in neuron B5, but probably not in neuron B19. The physiological role of NO released from neuron B5 is not known, however, in buccal neuron B2 of *Lymnaea*, NO has been found to mediate excitatory transmission onto surrounding neurons up to 50 μm away (Park et al., 1998). This neuron (B2) is believed to be homologous to buccal neuron to B5 in *Helisoma*. Therefore, in neuron B5, NO may also mediate excitatory neurotransmission between neurons within the buccal ganglia. Furthermore, neuron B19 is positioned very close to neuron B5 and could be affected by the release of NO.

Helisoma neurons have different states of neurite outgrowth

The rate of neurite outgrowth during neuronal development is vital for proper pathfinding of elongating growth cones, which must accurately grow to specific targets located hundreds or even thousands of microns away (Gallo and

Letourneau, 1999). Our results demonstrate that the rate of neurite outgrowth is regulated by a number of different factors, including extracellular calcium, cGMP, and NO. However, we have also found that the rate of neurite outgrowth varies between cells, and more surprisingly between neurites from the same cell. Furthermore, rate of outgrowth was an important determinant of the effect of NO and the other regulatory factors.

The difference in neurite outgrowth rates between neurons B5 and B19 may be due to intrinsic differences in calcium homeostasis. Previous studies have demonstrated that rate of neurite outgrowth of neurons B5 and B19 are not the same (Mattson et al., 1988; Cohan, 1992). Values between 13 um/hr and 17 um/hr were reported for the average rate of neurite outgrowth for neuron B19, whereas values between 15 um/hr and 26 μm/hr were reported for neuron B5. In the present study, we also observed a significant difference between these neurons; the average rate of neurite outgrowth was 15.2 ± 1.2 μm/hr for neuron B19, and 18.2 ± 0.3 μm/hr for neuron B5. The reason for B5's faster rate is not known, but may be due to the superior ability of neuron B5 to regulate $[Ca^{2+}]_c$. In comparison to neuron B19, neuron B5 can buffer increases in $[Ca^{2+}]_c$ more effectively (Mills and Kater, 1990). In theory, a strong regulator like neuron B5 would recover from the outgrowth inhibitory effects of a spontaneous calcium wave faster than a weak calcium regulator, like neuron B19, and advance at a faster average rate. Another possibility is that the basal $[Ca^{2+}]_c$ levels between these two neurons are different. Our data supports previous findings that neuron

B5 has a higher basal $[Ca^{2+}]_c$ than neuron B19 (Cohan et al., 1987), and this difference could account for the faster rate of outgrowth observed in neuron B5.

The difference in neurite outgrowth rates of neurons B5 and B19 could also be due to the presence of multiple populations of growing neurites within neuron B5. We have demonstrated for the first time that neuron B5 exhibits two different populations of growing neurites. One population displays a slow rate of neurite outgrowth, whereas the other displays a significantly faster rate. In contrast, only a single population of neurite outgrowth rates was observed in neuron B19. Furthermore, the population of slow-growing B5 neurites had a similar average rate of advance as the B19 neurites, suggesting that these two populations of neurites are in a similar state of outgrowth. The reason that neuron B5 typically exhibits a faster average rate of neurite outgrowth than neuron B19 is likely due to the presence of the fast growing population of B5 neurites. The origin of these fast growing neurites is not known, however, their occurrence suggests that neuron B5 contains a neurite outgrowth "switch" that can transform slow growing neurites into fast growers, or vice versa. This neurite outgrowth "switch" does not seem to be present in neuron B19.

The calcium hypothesis states that there is a window of $[Ca^{2+}]_c$ where neurite outgrowth is permissible, and within this window there is a $[Ca^{2+}]_c$ where outgrowth is optimal (Kater and Mills, 1991). A possibility exists where slow growing B5 neurites are within the permissible $[Ca^{2+}]_c$ window but are not at the

optimal $[Ca^{2+}]_c$, whereas fast growing neurites are at the optimal $[Ca^{2+}]_c$. A sufficient increase in $[Ca^{2+}]_c$ could propel a slow growing neurite to the optimal $[Ca^{2+}]_c$ and transform that neurite into a fast grower. This "switch" could be caused by a number of different factors, including extracellular cues or changes in calcium homeostasis. *Helisoma* neurons B5 and B19 exhibit state-specific differences in calcium homeostasis (Mills and Kater, 1990). Stable, non-growing neurites exhibit high calcium regulating abilities, whereas growing neurites exhibit reduced calcium regulating abilities (Mills and Kater, 1990). It is possible that the fast and slow growing B5 neurites identified in the present study exhibit different rates of neurite outgrowth because of differences in their ability to regulate calcium homeostasis.

NO enhances the rate of neurite outgrowth through a cGMP-independent mechanism

NO has been found to either inhibit or enhance neurite outgrowth in a number of different cell types (Hindley et al., 1997; Hess et al., 1993). In this study we have also observed both inhibitory and stimulatory effects of NO on neurite outgrowth. Furthermore, our data suggest that there are separate pathways for NO mediated stimulation and inhibition of outgrowth. Although our

results apply strictly to *Helisoma* neurons, they can provide insight into how other systems might function.

The NO-induced acceleration of neurite outgrowth in neurons B5 and B19 provides evidence that NO has a role in growth cone pathfinding. Typically, extracellular cues that enhance neurite outgrowth also guide neurites to their proper destinations in development by supplying attractive chemotropic gradients (for review see Kennedy and Tessier-Lavigne, 1995). Gradients of neurotrophic factors as well as neurotransmitters have been found to induce attractive growth cone turning in *Xenopous* spinal neurons (Ming et al., 1997; Zheng et al., 1994). Our results suggest that NO could also act as an attractive cue in the neuronal development of *Helisoma*.

The "state" of neurite outgrowth of neuron B5 neurites determines the effect that NO has on their rate of outgrowth. Slow growing B5 neurites and B19 neurites appear to be in a similar state of outgrowth, and upon application of NO switch to another state of outgrowth characterized by a significantly higher rate of elongation. However, slow growing B5 neurites respond slightly differently than B19 neurites. Slow growing B5 neurites demonstrate an inhibitory effect of NO that significantly attenuates the primary outgrowth enhancing effect on NO. Blockade of this inhibitory effect by reducing gyanylyl cyclase activity resulted in a NO-mediated increase in neurite outgrowth as large as that observed in B19 neurites.

Fast growing B5 neurites are in a different state of outgrowth than slow growing B5 neurites and B19 neurites, and respond to NO with a decrease in their rate of neurite outgrowth that is dependent on cGMP. Fast growing B5 neurites advance at a maximal rate of outgrowth. Therefore, it was not surprising that application of NO in the presence of a guanylyl cyclase inhibitor did not accelerate the rate of outgrowth of fast growing B5 neurites. Furthermore, fast growing B5 neurites advance at a greater rate than NO-stimulated slow growing B5 neurites and B19 neurites. These results suggest that different states of outgrowth respond to NO in different ways and that NO has the ability to transform neurites into new states of outgrowth.

NO increased the rate of neurite outgrowth in neurons B5 and B19, despite increases in $[Ca^{2+}]_c$ that are typically inhibitory. Previous studies indicated that increases in $[Ca^{2+}]_c$ in neuron B19 as little as 10 nM should inhibit neurite outgrowth (Torreano and Cohan, 1997). However, in the present study, NO increased $[Ca^{2+}]_c$ by an average of 39 nM, yet still accelerated neurite outgrowth in neuron B19. Furthermore, NO increased $[Ca^{2+}]_c$ in neuron B5 by an average of 166.8 nM, yet slow growing neurites still sped up. Our results suggest that NO has a stimulatory effect on neurite outgrowth that can accommodate for increases in $[Ca^{2+}]_c$ that are typically inhibitory. Perhaps this paradox could be explained by considering the source of the rise in $[Ca^{2+}]_c$ induced by NO. Our experiments with calcium-free medium and calcium channel

blockers suggest that intracellular calcium stores may contribute to the rise in $[Ca^{2+}]_c$. Thus, calcium released from intracellular stores in a specific spatiotemporal pattern may stimulate outgrowth promoting signaling pathways, which are normally inhibited by extracellular calcium influx.

In the continued presence of NO, B5 and B19 neurites are transformed into new states of outgrowth. However, upon removal of NO only B19 neurites recover to their original state of outgrowth. Slow growing B5 neurites do not exhibit a recovery to NO after washout with fresh defined medium, and continue to advance at a heightened rate. This suggests that NO transforms slow growing B5 neurites into a faster state of outgrowth. NO may change the state of outgrowth of slow growing B5 neurites by increasing the basal $[Ca^{2+}]_c$ of these neurites. B5 neurons exhibit a much larger NO-mediated increase in $[Ca^{2+}]_c$ than B19 neurons, and this increase could account for the change in outgrowth states observed in B5 neurites upon removal of NO. Large increases in $[Ca^{2+}]_c$ have been found to activate calcium regulating mechanisms in *Helisoma* neurons that are normally inactive (Kater and Mills, 1991). As NO causes a large increase in $[Ca^{2+}]_c$ in B5 neurons, it may also change the calcium regulating abilities of the neurites and transform them into a new state of outgrowth. This hypothesis is supported by the finding that slow growing B5 neurites bathed in calcium-free medium recover to pre-stimulation rates of outgrowth after NO is removed.

The NO-induced increase in neurite outgrowth was independent of cGMP production and extracellular calcium influx. As neuron B19 has previously been found to contain very little soluble guanylyl cyclase (Van Wagenen and Rehder, 2001), application of NO should not produce significant amounts of cGMP. Neuron B5, however, has been found to contain high levels of soluble guanylyl cyclase, and should therefore respond to NO with an increase in cGMP (Van Wagenen and Rehder, 2001). Furthermore, cGMP production appears to inhibit neurite outgrowth in B5 neurites. Taken together, we conclude that extracellular calcium influx and the cGMP transduction pathway do not mediate the neurite outgrowth-promoting actions of NO.

NO inhibits neurite outgrowth through a cGMP-dependent mechanism

Increases in $[Ca^{2+}]_c$ typically mediate decreases in the rate of neurite outgrowth of *Helisoma* neurons (for review see Mills and Kater, 1991). Furthermore, NO has been found to mediate large increases in $[Ca^{2+}]_c$ in neuron B5, as well as changes in filopodial morphology that resemble calcium induced F-actin bundle loss and growth cone stopping (Van Wagenen and Rehder, 1999; Welnhofer et al., 1999). All of these findings suggest that NO should mediate an inhibition of neurite outgrowth in neuron B5. However, as demonstrated in our study, NO only inhibits the minority of B5 neurites that are growing rapidly.

NO induces drastically different responses in fast and slow growing B5 neurites, and the cause for this may be due to differences in basal $[Ca^{2+}]_c$. We propose that fast growing B5 neurites have a $[Ca^{2+}]_c$ in the optimal level, whereas slow growing neurites have a $[Ca^{2+}]_c$ below the optimal level. Therefore, upon application of NO, the resulting $[Ca^{2+}]_c$ for fast growing neurites rises beyond the optimal level, and a reduction in the rate of outgrowth occurs. $[Ca^{2+}]_c$ also rises in slow growing B5 neurites, however the inhibitory effect of this increase is not sufficient to over-ride the enhancing effect of NO previously mentioned.

Fast growing B5 neurites do not recover from NO and are thus transformed into slow growers. A poor recovery was also observed in slow growers after washout of NO, and suggests that B5 neurites undergo a change in outgrowth state after treatment of NO. This change may be due to an alteration of basal $[Ca^{2+}]_c$, as suggested earlier for slow growers. To test this hypothesis, $[Ca^{2+}]_c$ should be compared between fast and slow-growing B5 neurites, both before and after the introduction of NO.

The inhibition of neurite outgrowth in fast growing B5 neurites was dependent on cGMP production and extracellular calcium influx. Previous findings have demonstrated that cyclic nucleotides depolarize neuron B5 and increase $[Ca^{2+}]_c$ (Van Wagenen and Rehder, 1999; Mattson et al., 1988). Furthermore, the NO-mediated increase in $[Ca^{2+}]_c$ in neuron B5 is dependent on cGMP production (Van Wagenen and Rehder, 1999). In the present study, we

found that a cGMP dependent mechanism is responsible for NO-mediated inhibition of neurite outgrowth. Combined, our results suggest that NO stimulates cGMP production in neuron B5 and mediates an influx of extracellular calcium, which has an inhibitory effect on neurite outgrowth. This pathway is not present in B19 neurons.

How does NO affect neurite outgrowth?

Nitric oxide is a membrane permeable gas that acts through many different mechanisms. It is for this reason that is it can cause contrasting effects in different cell types and even within the same cell. There is a large degree of discussion in the field of neurite outgrowth as to the effect of NO on growth cone morphology and neurite elongation. There are contrasting reports indicating that NO increases filopodial length (Van Wagenen and Rehder, 1999), inhibits neurite outgrowth (Hess et al., 1993), induces growth cone collapse (Ernst et al., 2000), and enhances neurite outgrowth (Hindley et al., 1997). It is quite clear that NO regulates neurite outgrowth, but it is not clear how this is accomplished. We suggest that there are two separate pathways activated by NO, and the effect of NO on neurite outgrowth depends on the contribution of both of these pathways.

It is well established that NO activates soluble guanylyl cyclase and increases cGMP levels (Mayer et al., 1990). Therefore, in all cells containing NO sensitive guanylyl cyclase, a cGMP-mediated signaling pathway can provide a

common mechanism by which NO may influence neurite outgrowth. In *Helisoma* neurons, cGMP causes neuron depolarization and calcium dependent regulation of growth cone morphology (Van Wagenen and Rehder, 1999; 2001; Price, 1994), probably by binding directly to a receptor and opening cyclic nucleotide gated channels, or possibly by activating protein kinase G (PKG), which can initiate numerous signaling pathways (Lucas et al., 2000). Furthermore, in *Xenopus* spinal neurons the level of cGMP can convert the response of growth cones to semaphorin from repulsion to attraction (Song et al., 1998). Therefore, cGMP not only has direct effects on neurite outgrowth, but intracellular levels of cGMP can modulate the directional behavior of growth cones in response to extracellular cues. However, cGMP has been found to mediate many contrasting effects on neurite outgrowth including growth cone collapse, turning, branching, and elongation (Campbell et al., 2001; Hindley et al., 1997). As we have demonstrated that cGMP has an inhibitory effect on neurite outgrowth in *Helisoma* neurons, we have provided a model to further study the role of cyclic nucleotides on neurite outgrowth.

The outgrowth-stimulating actions of NO are mediated by an unknown signal transduction pathway. One of the most likely candidates is a peroxynitrite/PKC/MAPK pathway. NO has been found to activate this pathway in rabbit cardiomyocytes (Balafanova et al, 2002), PC12 cells (Yamazaki, 2001; Jope et al., 2000), human T cells (Lander et al., 1996), rat fibroblasts (Bapat et al., 2001), rat colonic mucosal cells (Tepperman et al., 1999), smooth muscle

cells (Komalavilas et al., 1999), and human dopaminergic neurons (Oh-hashi et al., 99). The broad nature of this activation suggests that this action of NO is relatively conserved throughout cell types. However, the concentration of NO may be important in determining whether or not PKC is activated or inhibited. One group has found that peroxynitrite inhibits PKC activity (Knapp et al., 2001), but this group may have been using much higher concentrations of peroxynitrite than produced by NO released from donors or NOS. Regardless, in a number of systems NO/peroxynitrite acts to alter tyrosine residues on PKC to either activate or inhibit its activity. PKC and MAPK both have important regulatory functions on neurite outgrowth (Doherty et al., 2000), and regulation of their activity by NO could very well be the mechanism responsible for NO's neurite outgrowth enhancing effects.

Our results have demonstrated that NO acts to enhance neurite outgrowth downstream of $[Ca^{2+}]_c$, possible by stimulating the very cellular components activated during optimal $[Ca^{2+}]_c$. We suggest that the effect of NO is downstream of $[Ca^{2+}]_c$ because the observed increase in neurite outgrowth occurs during a typically inhibitory increase in $[Ca^{2+}]_c$. Calcium is proving to be a regulator of neurite outgrowth throughout many different systems, however, not all neurons are sensitive to increases in $[Ca^{2+}]_c$. Increasing $[Ca^{2+}]_c$ causes a reversible inhibition of neurite outgrowth in *Helisoma* neurons, hippocampal neurons (Song et al., 1994), locus coeruleus neurons (Moorman et al., 1994), dorsal root ganglia neurons (Fields et al., 1993; Lankford and Letourneau, 1991), and rat sensory

neurons (al-Mohanna et al., 1992. This inhibition is mediated by the actions of various compounds, including the calcium binding protein calmodulin (Polak et al., 1991), the calcium dependent protein phosphatase calcineurin (Lautermilch and Spitzer, 2000), and the calcium dependent proteinase calpain (Song et al., 1994). However, increasing $[Ca^{2+}]_c$ does not inhibit neurite outgrowth in all cell types. For example, in chick dorsal root ganglion growth cones, increasing $[Ca^{2+}]_c$ has no effect on filopodial length (Bonsall and Rehder, 1999). Furthermore, in rat sympathetic neurons, increasing $[Ca^{2+}]_c$ by up to 500 nM has no effect on neurite outgrowth (Garyantes and Rehder, 1992). Clearly $[Ca^{2+}]_c$ is a major signaling molecule for the regulation of neurite outgrowth, but its effect is dependent on other signaling molecules like calmodulin, calcineurin, and calpains. By directly influencing these downstream mediators it is possible to diminish or remove the effect of $[Ca^{2+}]_c$ on neurite outgrowth. Therefore, it is possible that NO acts in this way, in order to stimulate neurite outgrowth during increases in $[Ca^{2+}]_c$.

What Does NO Do *in vivo*?

The production of NO has been found to occur immediately after nerve injury in the leech CNS (Kumar et al., 2001). Whereas increases in NO measured with a citrulline assay have demonstrated the production of NO 30 min after injury, a direct assay of NO using a microsensor has recently demonstrated that it is produced seconds after nerve injury (Kumar et al., 2001). The

production of NO at the site of injury causes microglial accumulation, and aids in the immune response (Chen et al., 2000). Furthermore, NO may also act as an antioxidant to prevent further damage caused by reactive oxygen species generated during the injury (for review see Chiueh, 1999). As NO is produced immediately after nerve injury it aids in the immune response mounted against the damaged cells, however, our results provide a mechanism by which NO may also contribute to the regeneration of the damaged nerve. Increasing the rate of neurite outgrowth of damaged nerves could lead to an improved recovery of nerve function.

NO is known to play a role in the regeneration of damaged nerves in a number of different systems including optic nerves in the fish visual system (Devadas et al., 2001), motoneurons in the lizard tail (Cristino et al., 2000a; 2000b) olfactory receptor neurons in the rat epithelium (Roskams et al., 1994), rat paraventricular and supraoptic neurons (Wu and Scott, 1993), and rat dorsal root ganglion neurons (Gonzalez-Hernandez and Rustioni, 1999). The expression of NOS is upregulated for weeks following nerve injury, during the regeneration of neurons in the systems listed above. For example, in the lizard *Gekko gecko*, tail amputation is followed by rapid regeneration of the tail tissues and a growth of undamaged spinal nerves into the regrown tail (Cristino et al., 2000a; 2000b; 2000c). The spinal motoneurons and dorsal root ganglion cells that innervate the new tail upregulate their NOS, GAP-43, and cell death repressor protein Bcl-2 expression during axon elongation (Cristino et al., 2000c).

Increases in NOS and GAP-43 expression are also observed in regenerating dorsal root ganglion cells (Gonzalez-Hernandez and Rustioni, 1999), and NO has been found to increase GAP-43 expression in regenerating retinal ganglion cells (Klocker et al., 2001). Combined, this data suggests a role for NO in stimulating growth enhancing proteins and axon elongation during nerve regeneration *in vivo*. Furthermore, our data provide *in vitro* support that NO aids in regeneration by increasing neurite outgrowth of regenerating neurons. However, our results also suggest that NO-mediated cGMP production may inhibit nerve regeneration. Understanding how nitric oxide stimulates and inhibits neurite outgrowth could lead to new treatments for spinal cord damage and degenerative diseases.

The widespread expression of NOS in developing nervous systems has lead to an examination of the role of NO in neuronal development. NOS expression has been found in the nervous systems of developing molluscs (Serfozo et al., 1998; Lin and Leise, 1996; Goldberg et al., 1999), insects (Seidel and Bicker, 2000; Ball and Truman, 1998), amphibians (Moreno et al., 2002; Lopez and Gonzalez, 2002), rodents (McCauley et al., 2002) and humans (Foster and Phelps, 2000). The effect of NO on development has been closely examined in the visual system, where NOS knockout mice exhibit a delayed development of the ipsilateral retinocollicular pathway (Wu et al., 2000). Moreover, NO has been found to refine visual connections during retinal ganglion cell axon arbor remodeling in rats (Cogen and Cohen-Cory, 2000), and is

required for the formation of proper retinal synapses in *Drosophila* (Gibbs et al., 2001).

Our results have demonstrated that NO regulates neurite outgrowth, and suggests that the developmentally regulated expression of NOS could influence neuronal development. This is supported by numerous findings linking changes in NOS expression to developmental changes like metamorphosis (Lin and Leise, 1996), axon arbor refinement (Cogen and Cohen-Cory, 2000) dendritic branching (McCauley et al., 2002) and synapse formation (Wright et al., 1998). In *Helisoma*, NOS activity in developing neurons helps regulate neuronal branching and morphology (Goldberg et al., 99). Furthermore, the expression of NOS appears to be developmentally regulated in *Helisoma* embryos. The production of NO during neuronal development is likely an important signaling cue for growth cone pathfinding. Concentration gradients of NO could act as an attractive guidance cue for advancing growth cones that respond to NO with an increase in their rate of outgrowth, like B19 and slow growing B5 neurites. Furthermore, the ability of NO to promote neurite outgrowth during increases in $[Ca^{2+}]_c$ suggests that NO could allow specific growth cones to advance through extracellular gradients of inhibitory guidance cues. However, all of these effects are dependent on the production of NO, which is dependent on the regulated expression of NOS.

It is becoming clear that NO has widespread effects on different types of cells and as shown in our study even on the same cell. We have demonstrated that NO can regulate neurite outgrowth in a cGMP-dependent and independent fashion. The implications of our findings are widespread and will hopefully lead to further research into neuronal development and spinal cord injury. Future research needs to be done to determine the pathway(s) responsible for NO's inhibitory and excitatory effects on neurite outgrowth. Further understanding of these pathways could lead to promising new treatments for spinal cord injury and greater insight into the wonders of neuronal development.

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